Exploiting fungal cell factories for pigment production

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Exploiting fungal cell factories for pigment production

Ph.D. dissertation by Gerit Tolborg

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Preface

With this thesis, I conclude my time as a Ph.D. student at the Technical University of Denmark (DTU) in the period from December 2012 to June 2018, which was funded by DTU. The project was initially supervised by Ulf Thrane and by Jette Thykær. When Jette Thykær left DTU in 2014, Mhairi Workman took over supervision of the fermentation part of the project. In 2016, both Mhairi Workman and Ulf Thrane quit DTU and Thomas Ostenfeld Larsen (main supervisor) and Anders Ødum (co-supervisor) became associated with the project.

This thesis is divided into 8 chapters. **Chapter 1** presents the general motivation for research in discovery of novel fungal pigments. **Chapter 2** provides some theoretical background to support the following chapters. This includes a brief introduction into natural colorants, filamentous fungi and their secondary metabolites, as well as *Monascus* Pigments and fungal cell factories. **Chapter 3** details the overall experimental procedures for propagation of the fungus, for bioreactor set-up, for media preparations and for general chemical analysis protocols. Additionally, each chapter contains a small experimental section with procedures exclusively relevant for the individual chapter. **Chapter 4-7** present the main results including a brief introduction at the beginning of each chapter. **Chapter 4** screens different media compositions for their effect on pigment production in *T. atroroseus* and **Chapter 5** investigates different process parameters. **Chapter 6** describes the discovery of the novel class of azaphilone pigments called atrorosins. **Chapter 7** presents a process for the selective production of single atrorosins using *T. atroroseus* as a fungal cell factory. **Chapter 8** provides an overreaching conclusion across all chapters and also contains future perspectives. Unless otherwise stated, all tables, figures and schemes have been prepared by me. A detailed list of abbreviations used throughout the dissertation can be found in Table A1 on p.133. This thesis is the culmination of many years of research and I hope that you will enjoy the expedition into the chart of color of fungal pigments.

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Tin-containing Silicates for Carbohydrate Conversion

June 2018

Acknowledgements

There are many people I would like to thank for making my time as Ph.D. student at DTU a great experience. This includes people who have taken part in the work done, who have supported me or who have simply been there to make the hours in the lab more enjoyable.

During my years as a Ph.D. student, I had the honor of giving birth to two wonderful sons. Eskild was born in 2014, and Levin in 2017. Becoming a mother, always puts things in a new perspective and in my case also elongated my time as a Ph.D. student to a total of six years. I saw many people come and go. Our department changed name from DTU Systems Biology to DTU Bioengineering, and I stopped counting how many times the internal structure of our department was revised. These were disrupting years for DTU Bioengineering, that as a consequence let to several changes in supervisors- for me and others. During that time, it was very important to have anchors- both at work and at home. Anchors, who stay put, and wait with you until the storm is over.

First and foremost, I would like to thank all my supervisors I have had during the six years. Jette and Ulf got me started and settled in the world of pigments. In 2014, Mhairi took over the lead and initiated a very fruitful collaboration with Thomas Ostenfeld Larsen that advanced the project with regards to the analytical chemistry. When Mhairi found new challenges elsewhere in 2016, Thomas gladly became my new main supervisor, even though he has background in analytic chemistry. Together with Anders Ødum, who joined DTU in 2016, he supported me until this project finally reached the finish line. Anders was my daily motivator, my eye for detail, my bad conscience and my voice of reason, and has since he set foot into DTU Bioengineering, been a very precious colleague and friend.

During my years at DTU, I met so many great people, too many to mention them all here, but their assistance, their help and their support is no less appreciated. Anna-Lena Heins, Peter Boldsen Knudsen, Sietske Grijseels and Milica Randelovic have shared with me the joy of bioreactor cultivation over the years. Thomas Isbrandt helped giving the project an "chemistry" make-over and

without Tina Johansen and Martin Nielsen, who always helped when there were equipment issues in the lab, I would have burst into tears way more often than I actually did. I would like to thank Regina Åris Schürmann who helped me a lot with the chemostat cultivation and media preparation. Special thanks belongs also to Valéria De Carvalho Santos Ebinuma from UNESP in Araraquara, Brazil, who gave me a warm welcome in her lab during my external stay in 2016.

A good working atmosphere is always key for the motivation – so I would like to thank all my colleagues from Building 223 & 221. You have all contributed to making my workdays a little brighter.

At the end, I would like to thank my parents who let me follow my heart and move to Denmark. And, I would like to thank my husband Søren- who is a great inspiration, my daily accomplice and the center of my life. He has been a tremendous support and help- particularly in the writing process and he can do magic when it comes to formatting in Microsoft Word. He himself got a Ph.D. with great success in 2017 and has been mentioning it daily – ever since.

Finally, I can draw level.

Abstract

The growing concern over harmful effects of synthetic colorants has led to an increased interest in natural coloring alternatives. Currently, natural colorants are extracted from fruits or roots and their production is thus highly dependent on the supply of the extraction source, which can fluctuate heavily between seasons. To overcome limitations in supply, quantity and quality, alternative routes to securing a stable, sustainable production of natural colorants for food applications are required. Here, the use of fungal cell factories for pigment production is at the focal point of interest. *Monascus* Pigments are fungal pigments derived from *Monascus* species with yellow to red color hues. They usually occur in a plethora of different pigment compounds and their exact composition is thus hard to determine or to control.

Monascus Pigments have been used in the Asian food industry for centuries, however since their production is associated with the mycotoxin citrinin, *Monascus* Pigments are not approved for human consumption in neither Europe nor the USA. Different *Monascus* Pigment producing species - other than *Monascus* - have been described in the literature so far, one of them being *Talaromyces atroroseus*. *T. atroroseus* has recently been shown to excrete large amounts of *Monascus* Pigments, without production of mycotoxins. Some of the pigments are known, such as PP-O, PP-V, PP-Y, but the majority of the produced pigments have not been previously structurally characterized.

The major aim of this thesis is to propose new microbial cell factories for reliable pigment production. To do so, the potential of the red pigment producer *T. atroroseus* was thoroughly investigated.

Firstly, different media compositions were screened and it was shown that red pigment production of *T. atroroseus* was enhanced using KNO_3 as the nitrogen source and sucrose as the carbon source. A suitable cultivation medium was proposed and *T. atroroseus* was shown to be reproducibly cultivable in stirred tank reactors, which is a crucial requirement for industrial fungal cell factories.

In a second step, process parameters were investigated and it was demonstrated that pigment production *T. atroroseus* was heavily influenced by pH and temperature. Cellular performance in terms of fungal growth was enhanced at pH 3, but pigment production was greatly increased at a pH of 4.5. It was shown that there is a clear trade-off between propagation of *T. atroroseus* and pigment production since no tested value of pH supported both equally well. In order to promote growth and pigment production, a cultivation method comprising a pH switch from pH 3 to pH 4.5 was developed. By employing this method, pigment production by *T. atroroseus* could be increased by 35%.

Furthermore, in collaboration with DTU Natural Product Chemistry different chemical analysis tools were used to investigate the pigment production portfolio of *T. atroroseus*. Here, we confirmed that the strain IBT 11181 produces the known *Monascus* Pigment PP-V and for the first time, both the *cis*-and *trans*- form of PP-O were described.

Additionally, as a result of this collaboration, a new series of *Monascus* Pigment derivatives of the known azaphilone pigment PP-O has been characterized. These have been named atrorosins and have different amino acids incorporated into the azaphilone core.

Finally, a fermentation process tailoring pigment production in *T. atroroseus* was designed to selectively produce only one atrorosin at a time. By doing so, pigment purification was greatly simplified. *T. atroroseus* was cultivated with single amino acids as nitrogen source. The presence of an amino acid in excess promoted production of only the atrorosin derivative of that particular amino acid. All amino acids promoted growth of *T. atroroseus* but the pigment yield greatly varied and was best with histidine, serine and glutamate. To improve the set-up, a two-step fermentation process for production of the serine-derivative was developed. Here, in a first step nitrate limiting conditions promoted the production of *cis*- and *trans*-PP-O. In a second step, the amino acid serine was added to the cultivation medium, converting both PP-O isomers into *cis*- atrorosin-S. This process design yielded essentially pure *cis*- atrorosin-S and final pigment titers of 0.9 g/L were reached. To the best of our knowledge, for the first time, production yields of *Monascus* Pigments could be given in g/L and not in Absorbance Units.

Altogether, the work presented here, has established a stable, quantifiable and high yielding cultivation process for *T. atroroseus* and sets therewith the cornerstone in implementing pigment production in fungal cell factories.

Resumé

Den stigende bekymring over skadelige bivirkninger forårsaget af syntetiske farver har øget interessen for at finde naturlige alternativer. Nuværende naturlige farvestoffer er primært ekstraheret fra frugter og rødder; hvilket gør, at både kvalitet og tilgængelighed er svingende fra sæson til sæson. For at undgå begrænsning i forsyning, kvalitet og kvantitet og samtidig sikre en stabil og bæredygtig fremstilling af farvestoffer til fødevareindustrien er det nødvendigt at finde alternative produktionsmetoder. Af den grund er interessen for produktion af farvestoffer ved brug af cellefrabrikker baseret på svampe stærkt stigende.

En lovende svamp er arten *Monascus*, som producerer naturlige gule, orange og røde farver. *Monascus* pigmenter har i Asien været brugt til farvning af fødevarer i århundreder, men da arten forbindes med samproduktion af mykotoksinet citrinin er *Monascus* pigmenter ikke godkendt til anvendelse i EU eller USA. Alternative svampearter til fremstilling af disse *Monascus* pigmenter (udover *Monascus* svampen selv) er tidligere blevet identificeret og beskrevet. Én af disse er arten *Talaromyces atroroseus*, som tidligere har tilhørt slægten *Penicillium*. Den producerer *ikke* citrinin under produktion af disse farvestoffer og repræsenterer derfor et vigtigt, potentielt alternativ til *Monascus* arten. Ved gængse produktionsmetoder vil *Monascus* pigmenterne fremstå i en blanding, hvoraf kun et fåtal af de dannede farvestoffer er kendte (PP-O, PP-V, PP-Y), mens resten stadigvæk mangler at blive identificeret.

I denne Ph.d. afhandling bliver arten *Talaromcyces atroroseus* karakteriseret og grundigt undersøgt for dens evne til at producere nye, røde pigmenter i en sikker og reproducerbar proces.

Først blev flere mediesammensætninger screenet og efterfølgende blev enkelte komponenter i mediekompositionen grundigt undersøgt. Resultaterne demonstrerede, at ved brug af KNO_3 som kvælstofkilde og sakkarose som kulstofkilde blev produktionen af røde pigmenter betydeligt forhøjet. Sammensætningen af et velegnet kultiveringsmedium er i denne afhandling blev foreslået. Ydermere blev der vist god reproducerbarhed under kultiveringen af *T.*

atroroseus i bioreaktorer. Dette er særdeles vigtigt og en forudsætning for senere opskalering til industriel produktion.

Efterfølgende blev procesparametrene grundigt undersøgt, og det blev demonstreret, at pigmentproduktionen fra *T. atroroseus* er yderst afhængig af forhold såsom pH og temperatur. Effektiviteten af cellefabrikken i forhold til vækst og spredning var højest ved en pH på 3, imens at de højeste pigmentudbytter blev opnået ved en konstant pH på 4.5 og en temperatur på 30°C. Ud fra disse forsøg var det tydeligt, at der måtte ske en afvejning i forbindelse med produktionen imellem vækst i biomasse og produktion af farve, da ingen pH værdi understøttede begge vigtige aspekter af produktionen.

For at fremme og optimere produktionsprocessen blev en to-trins proces udviklet, der først favoriserede vækst af svampen og dernæst farveproduktionen. Denne fermenteringsproces blev designet med en indledende vækstfase med en konstant pH på 3 efterfulgt af en ændring af pH til 4.5. Denne metode øgede farveproduktionen fra *T. atroroseus* med 35%.

Ved hjælp af spektroskopi-baseret analyse foretaget i tæt samarbejde med Thomas Isbrandt fra DTU Natural Product Chemistry blev det undersøgt hvilke specifikke farvestoffer, der blev produceret ved anvendelse af *T. atroroseus*. Det blev bekræftet, at arten IBT 11181 producerer den kendte *Monascus* pigment PP-V, men det blev samtidig også vist for første gang, at både en *cis*- og en *trans*-form af pigmentet PP-O blev dannet. Yderligere blev en helt ny serie af *Monascus* pigmenter identificeret og karakteriseret til at være derivater af det kendte azaphilon-pigment PP-O. Disse nye pigmenter blev navngivet 'atrorosiner' og er kendetegnet ved at have en aminosyre inkorporeret i azaphilon-strukturen.

Til slut blev en fermenteringsproces baseret på *T. atroroseus* designet til selektivt kun at producere ét udvalgt atrorosin pigment i stedet for den blanding af *Monascus* pigmenter, som traditionelt har været slutproduktet. En sådan selektiv proces vil kunne simplificere oprensning og analyse betydeligt, da forskellige pigmenter ikke skal separeres. Ved at anvende en enkelt aminosyre som kvælstofkilde under fremstillingen blev det vist, at denne aminosyre selektivt indsættes i PP-O under kultiveringen og derved fører til dets tilsvarende atrorosin pigment. De tyve gængse aminosyrer blev testet enkeltvis. Alle kunne understøtte vækst af *T. atroroseus,* men ikke alle aminosyrer kunne understøtte farveproduktion i rystekolber. *Produktionen var best med histindine, serine og glutamate.* For at optimere produktionen blev en to-trins proces herefter designet for processen med serine som aminosyre. I det første trin blev KNO_3 tilføjet som nitrogenkilde i underskud for at fremme dannelsen af *cis*- og *trans*-PP-O. I det andet trin blev serine tilføjet til kultiveringsmediet, hvilket forårsagede en omdannelse af begge PP-O isomerer til *cis*-konfigurationen af serine atrorosinen, *cis*-atrorosin-S. Denne optimerede

proces resulterede i næsten udelukkende fremstilling af *cis*-atrorosin-S med et endeligt udbytte på 0,9 g/L.

Igennem alle rystekolbe- og bioreaktor forsøg med *T. atroroseus* blev der undersøgt for citrinin produktion, og da det ikke blev observeret, kan det konkluderes, at *T. atroroseus* egner sig markant bedre end *Monascus* som en industriel produktionsorganisme.

Igennem denne Ph.d. afhandling er en stabil, reproducerbar kultiveringsproces af *T. atroroseus* blevet udviklet med høje opnåede pigmentudbytter. Dette giver et godt udgangspunkt for implementering af *T. atroroseus* i en fremtidig industriel proces.

Contents

1 Motivation of the project*

Today, one of the most distinctive features of manufactured food is its colorful visual perception, and the purchase behavior of modern consumers is frequently driven by appearance. In the supermarket, a particular color is often associated with freshness and quality of the product. At the same time, consumer awareness increases over diet and health, and the potentially harmful effects of synthetic colorants. The rapid growth of sectors like processed foods together with stringent regulations demanding limited use of synthetic colors, challenges the food additive industry in providing natural color alternatives and in replacing chemically synthesized dyes with bio-derived ones.

The natural food colorant market in Europe was estimated to a value of USD 600 million in 2016 and was expected to grow at a compound annual growth rate of over 6% to be worth over USD 1 billion by 2025 (Grand view Research, 2018).

In 2014, for the first time, the market of natural colorants drew level with the market share of synthetic colorants as both represented 34% of the overall food color market (Figure 1.1) (Grand view Research, 2014). Key applications of food colors are confectionary and beverages. Other applications include packaged foods, dairy products, frozen foods, condiments, dressings, functional food and pet food.

The topic of synthetic dyes in food has been the subject of debate for many years (Kobylewski & Jacobson, 2010) and it seems that natural or nature-identical colorants have a more healthy image in the eye of the consumer (Downham &

* Parts have been adopted from Tolborg *et al.* (2017). Establishing novel cell factories for producing natural pigments in Europe. In O. V. Singh (Ed.), *Biopigmentation and Biotechnological Implementation* (First Edit, pp. 23–60). Wiley, NJ, USA.

Collins, 2000). A recent survey in the US found that 62% of consumers usually seek out products with a "natural" food label (Consumer Reports® national Research Center, 2015). It appears thus evident that consumer demand for natural food colorants and transparent labeling will be major drivers for facilitating growth of the natural food color sector.

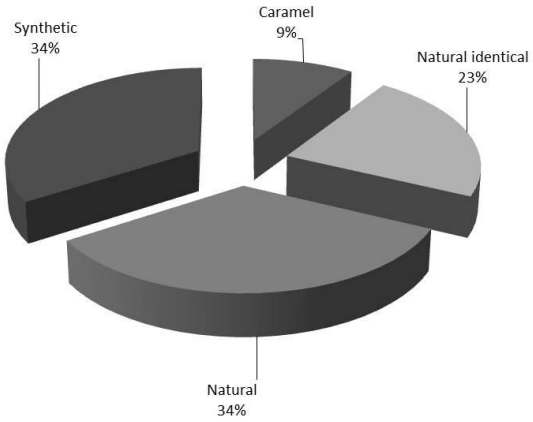


Figure 1.1. Percentage market share of food colorants in 2014 (Grand view Research, 2014)

Currently, most natural colorants are extracted from fruits and roots making their production dependent on the supply of raw materials with large variations in quantity and quality (Wissgott & Bortlik, 1996). These limitations could be overcome by employing the techniques of industrial biotechnology by cultivating, suitable, robust microbial hosts ("cell-factories") in industrial scale fermenters (Tolborg et al., 2017). Fungi, particularly ascomycetes and basidiomycetes, are known to naturally biosynthesize and excrete diverse classes of secondary metabolites (SM) including pigments, such as carotenoids, melanins, flavins, and quinones (Dufossé et al., 2005; F. A. E. Torres et al., 2016). So far, this potential has remained more or less untapped, however the successful use of fermentation technologies could allow the efficient production of fungal pigments (Dufossé, 2006). Up till now, there are only limited reports on bioprocesses producing fungal pigment on industrial scale (Dufossé et al., 2014). One example is the application of species within the fungal genus *Monascus*.

Solid state fermentation of rice by *Monascus* has a long tradition in East Asian countries dating back at least to the first century A.D (Feng et al., 2012). *Monascus*

fermented rice products are called "ang-kak" and are used as a food colorant for yellow, orange, and red color hues (Arunachalam & Narmadhapriya, 2011). *Monascus* Pigment production is however associated with the harmful mycotoxin citrinin and as a result these pigments are not approved for human consumption in Europe and the USA (Mapari et al., 2010).

The implementation of new fungal microbial cell factories for reliable color production is important for the advancement of safe, biobased alternatives to chemically synthesized pigments for the European Market.

In order to commercialize fungal colorants, their safety will have to be rigorously assessed and the strict requirements for novel food by the European Food Safety Agency (EFSA) will have to be fulfilled.

The recently discovered producer of *Monascus*- like pigments, *Talaromyces atroroseus* could serve as an potential candidate, , representing a safe alternative to existing natural red colorants (Frisvad et al., 2013). A solid knowledge of cellular performance would allow pigment production in *T. atroroseus* to be optimized and implemented on industrial scale. For that, firstly, standardized cultivation protocols are needed for the establishment of reproducible processes. Secondly, analytical methods need to be developed in order to analyze and confirm known molecular pigment structures or to elucidate novel ones.

The motivation for this PhD thesis was thus the necessity for innovative biotechnological processes for natural pigment production together with the desire to explore the great potential of the newly identified *Monascus* Pigment producing strain called *T. atroroseus*.

1.1 Previous work conducted at DTU Bioengineering

This PhD project contributed to ongoing research of pigment producing filamentous fungi at DTU Bioengineering. In 2008, Sameer Mapari identified in his thesis "Chemotaxonomic Exploration of Fungal Biodiversity for Polyketide Natural Food Colorants- Discovery & Evaluation of cell factories and Characterization of Pigments" one promising pigment producer called *Penicillium purpurogenum*. In 2013, *P .purpurogenum* was reclassified as *T. atroroseus* and the first metabolic profile revealed no indication of mycotoxin production (Frisvad et al., 2013). In 2015, Kasper Bøwig Rasmussen fully sequenced the genome of *T. atroroseus* and created several knock- out strains. He also identified the *Monascus* Pigment derivative N-glutamyl monascorubraminic acid in *T. atroroseus* and so helped to further understand the pigment portfolio of this strain.

1.2 Process design for pigment production in *T. atroroseus*

The aim of this PhD project was to design a reproducible submerged process for red pigment production with the filamentous fungus *T. atroroseus* in bioreactors, including media recipe and cultivation condition. This also involved the development of an analytic tool for quantification of produced pigments as well as further elucidation of the pigment profile of *T. atroroseus* under different cultivation conditions. Throughout my Ph.D., I closely collaborated with Thomas Isbrandt from DTU Natural Product Chemistry, who conducted most of the chemical analysis presented in this thesis.

2 Theoretical†

This chapter summarizes the theoretical background relevant for the presented work. This includes a brief introduction into colorants, secondary metabolites of fungi and polyketides as well as the presentation of fungal pigments derived from *Monascus*. Furthermore different parameters for cell factory design are introduced and put into context with fungal production of *Monascus* Pigments.

† Parts have been adopted from Tolborg *et al.* (2017). Establishing novel cell factories for producing natural pigments in Europe. In O. V. Singh (Ed.), *Biopigmentation and Biotechnological Implementation* (First Edit, pp. 23–60). Wiley, NJ, USA.

2.1 Colorants

2.1.1 Classification of colorants

A colorant is a substance that is added in order to change the color of a material, a surface or a solution. Colorants can be either dyes or pigments. These terms are, often used interchangeably for substances responsible for coloration of the medium within which they are applied. The difference between the two is that dyes are soluble in the applied medium and pigments are not. Hence a colorant can change from being a dye to being a pigment. For example carotenoids are dyes in oil but pigments in water. For biological pigments this distinction is normally not used and all colorants are referred to as pigments (Delgado-vargas, 2003).

Pigments are compounds capable of absorbing visual light. Reflected light is observed by the human eye as the color of the light not absorbed. The distribution of light as electromagnetic radiation, according to either energy or frequency of wavelength, is known as the electromagnetic spectrum. The part of the electromagnetic spectrum covering visible light and the color it is associated with are illustrated in Figure 2.1 (modified from College, 2012).

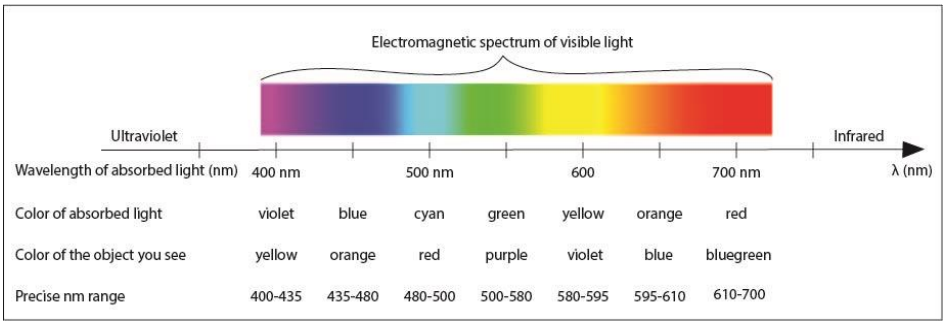


Figure 2.1 Part of the electromagnetic spectrum including visible light that explains the difference between absorbed and observed light.

The ability for pigments to absorb light and thereby appear colorful is linked to the chemical structure of the compound, more specifically to the system of conjugated double bonds, known as the chromophore. When double bonds appear in a conjugated system, electrons can delocalize across the system of overlapping *p*-orbitals. When irradiated by light, the electrons can absorb photons of specific wavelengths depending on the size of the conjugated system. As a result, only some wavelengths are reflected leading to the colored appearance of

the molecule, *e.g.* at 500 nm, the color of the absorbed light is cyan leading to a red color of the object in question (see Figure 2.1).

Food colorants can be categorized as natural, nature-identical and synthetic colorants. Natural colorants are pigments that are found in nature as they are biosynthesized by a living organism. They are mainly plant extracts or pigments from plants, *e.g.* red from paprika or beetroots; yellow from saffron; orange from annatto; green from leafy vegetables (Dufossé, 2006). Nature-identical colorants are chemically synthesized pigments, with identical chemical structures to colorants found in nature, examples include β-carotene (orange) and betanin (red). This group of colorants also includes pigments resulting from chemical modifications of natural colorants. Artificial colorants are purely man-made and do not occur in nature (Scotter, 2011). Artificial colorants such as Allura Red, Brilliant Blue and Sunset Yellow are still widely used in the industry as coloring agents in cosmetics, drugs, candies, beverages and many other foods (Amchova et al., 2015). However, the number of permitted artificial colors has gradually been reduced over the last 30 years since several negative side effects have been associated with their consumption (Scotter, 2011).

Colorants which are used in the food industry need to comply with particularly strong standards and regulations. Legislation ensures that only certain pigments are permitted as food colorants and compound-specific purity standards are subject to strict regulation. Quality control of the pigments should also be a strict requirement but is mainly related to color strength, hue, and intensity where certain criteria have to be met (Tuli et al., 2015). If microorganisms were to produce food pigments, the absence of pathogens and mycotoxins would have to be rigorously verified. Other favorable characteristics of food pigments are that they should be tasteless, odorless, and unreactive with other constituents of the food. They should also be stable over a wide range of pH and temperature, and insensitive to light or exposure to air (Mapari et al., 2005).

2.2 Secondary metabolites of filamentous fungus

Filamentous fungi produce a broad range of different secondary metabolites (SM) like mycotoxins, antibiotics or pigments (J.-H. Yu & Keller, 2005). The secondary metabolite profile of an organism depends strongly on the growth conditions and can vary a lot. SM are small organic compounds which are very diverse in their structures and functions. Their applications are many-fold and include immune suppressants, anti-tumor drugs, and enzyme inhibitors. The majority of SM are produced via enzymatic pathways after the fungus has completed its initial growth, meaning that SM do not play any essential role in basic cell development but rather give their producing organism beneficial

characteristics in a competitive ecological environment (Adrio & Demain, 2003). Fungal SM can be divided into four main chemical classes: polyketides, terpenoids, shikimic acid derived compounds, and non-ribosomal peptides (Pusztahelyi et al., 2015). In the scope of this thesis, only polyketides will be discussed further.

2.2.1 Polyketides

Polyketides are the most abundant fungal secondary metabolites and they are synthesized by multi-domain enzymes called polyketide synthases (PKS) (Keller et al., 2005). Fungal PKS are iterative and synthesize polyketides by condensing short-chain carboxylic acids to form carbon chains of various lengths (Keller et al., 2005). The group of fungal polyketides includes bioactive compounds (lovastatin), mycotoxins (citrinin) and pigments such as anthraquinones (Gessler et al., 2013), naphtoquinones (Medentsev & Akimenko, 1998) and azaphilones (Osmanova et al., 2010). Fungal pigments often possess certain bioactivities which give the fungus an advantage in natural ecological niches (Firn & Jones, 2003). Anthraquinone pigments apply a color (usually yellow, orange, or brown) to the mycelium of microscopic fungi or their fruiting bodies. In the textile industry, fungal anthraquinones are widely used as dyes (Gessler et al., 2013). The interest in naphthoquinone synthesis by fungi is related to the broad spectrum of their biological activity, including phytotoxic, insecticidal, antibacterial, and fungicidal properties. More than 100 structures of naphthoquinone metabolites produced by fungi of 63 species, of which most belong to the genus *Fusarium*, have been described (Medentsev et al., 2005). Azaphilones can be colored and non-colored compounds. They are produced by numerous species of ascomyceteous and basidiomyceteous fungi, including genera *Aspergillus*, *Penicillium*, *Chaetomium*, *Talaromyces*, and *Epicoccum*, as well as *Monascus* (Osmanova et al., 2010). They are responsible for bright yellow, red, or green colors of fruiting bodies or mycelia (Gao et al., 2013). Some examples of each group are shown in Figure 2.2. Some of the best known polyketide pigments from the group of azaphilones are called "*Monascus* pigments" and are produced by *Monascus* species (Feng et al., 2012).

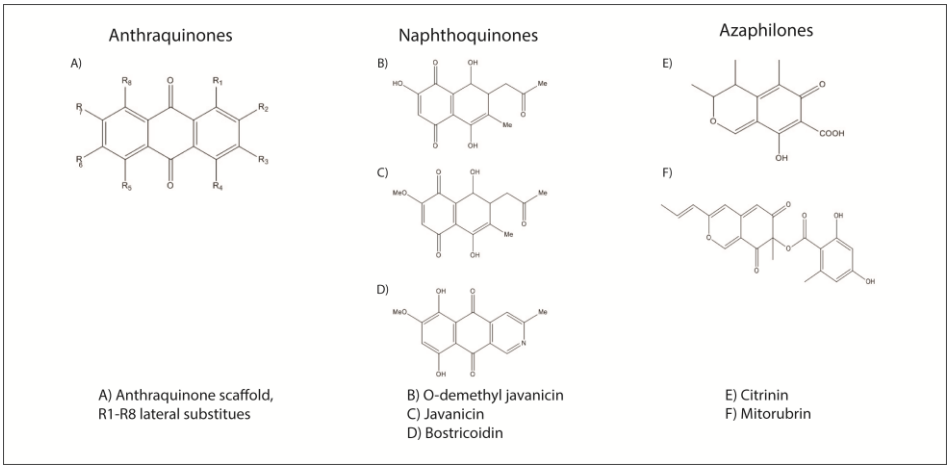


Figure 2.2 Chemical structures of examples from anthraquinones, naphthoquinones and azaphilones

2.3 *Monascus* Pigments

The term "*Monascus* pigments" (MP) refers to a mixture of azaphilones including yellow, orange, and red constituents. This group of pigments was first described in association with *Monascus* species, but numerous other species have been linked with their production (see Table 2.1 on p. 15) (Gao et al., 2013). Yellow and orange azaphilones are known for their ability to react with amines by exchanging the pyran oxygen with nitrogen (Stadler et al., 1995). This exchange leads to a color shift from yellow/orange to red (see Figure 2.3). Up until 1973, six classic MP were identified, namely monascin (F. C. Chen, 1969) and ankaflavin (Manchard & Whalley, 1973) (yellow, absorbance at 330-450 nm); rubropunctatin (Haws et al., 1959) and monascorubrin (Fielding et al., 1960; Kumasaki & Nakanishi, 1962) (orange, absorbance at 460-480 nm); and rubropunctamin and monascorubramin (Kumasaki & Nakanishi, 1962) (red, absorbance at 400 nm and 500 nm). Their chemical structure and UV-Vis spectra are illustrated in Figure 2.3. Aside from these six azaphilone pigments, more than 50 related pigments have been reported (Feng et al., 2012; Gao et al., 2013; Osmanova et al., 2010; Patakova, 2013). Numerous studies have been conducted on MP in regard to their structures as well as to their biosynthetic pathway, optimized production strategies, detection methods or their biological activity and great review articles are available *e.g.* Juzlova, Martinkova, and Kfen (1996), Feng, Shao, and Chen (2012) and Patakova (2013). Species used for industrial MP production include *M. pilosus*, *M. purpureus*, *M. ruber*, and *M. anka* (Feng et al., 2012).

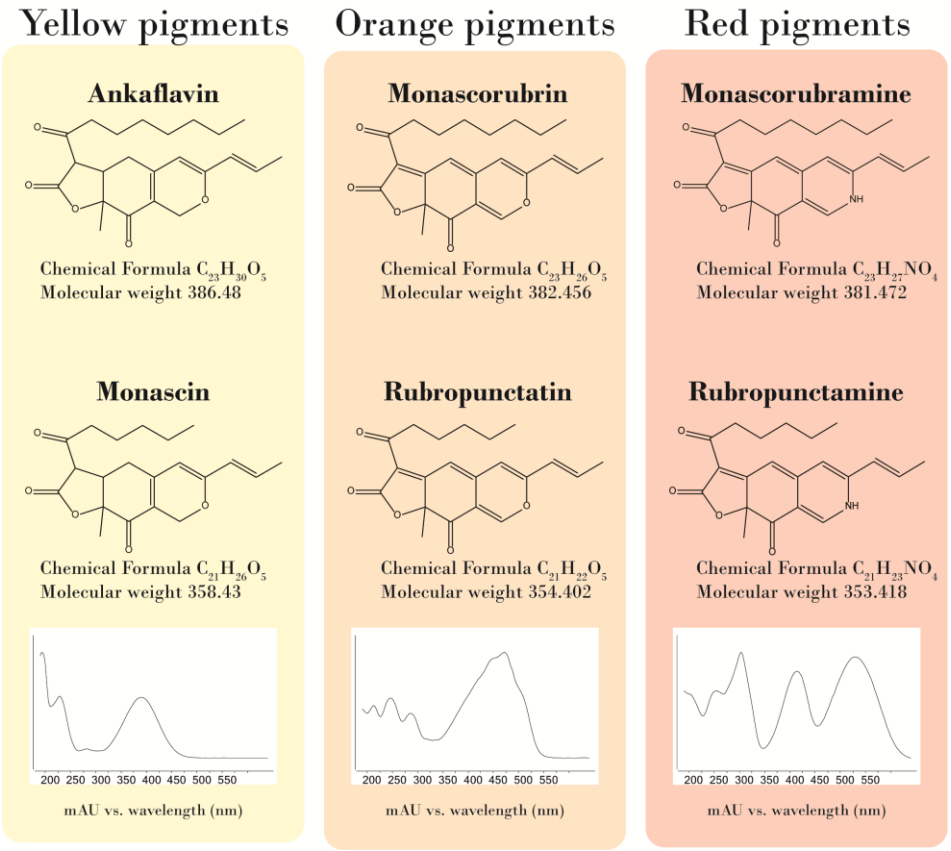


Figure 2.3 Chemical structures of the six classical *Monascus* pigments and their corresponding UV-Vis spectra

As mentioned previously, a large market for MP can be found in Asia. In Japan for instance, the annual consumption of MP has increased significantly over the last 30 years and new food applications for MP like the coloration of processed meats (sausage, ham), marine products like fish paste and tomato ketchup have been reported (Dufossé et al., 2005). However despite the enormous economic potential of MP, they have not yet found their way into the European market, due to the association of several species of *Monascus* with the mycotoxin citrinin (Blanc, Laussac, et al., 1995; Blanc, Loret, et al., 1995).

Citrinin production seems to be related to pigment production in *Monascus* spp. and can be influenced by both media and cultivation conditions (H Hajjaj et al., 1999; Wang et al., 2005; Zhang et al., 2013). In order to use *Monascus* species for food colorant production for the European market, non-citrinin producing

species or culture conditions unfavorable to citrinin production need to be developed. So far, several attempts have been successful in defining the conditions for citrinin-free MP production (D. Chen et al., 2016; Kang et al., 2014). However, once a microorganism has been associated with a specific mycotoxin, the application for FDA or EFSA approval can be a complex and elaborate process.

Instead, it seems more promising to screen for alternative and "citrinin-free" species, other than those from the *Monascus* genus, which produce MP, providing a safe and viable alternative for the European market (Mapari et al., 2005).

2.3.1 Biosynthesis of *Monascus* Pigments

In spite of the large-scale commercial production and their economic potential, the understanding of the biosynthesis of MP remains still incomplete. In 2017 Chen *et al.* contributed to the elucidation of the biosynthetic pathway in *Monascus ruber* M7 by a combination of targeted gene knockouts, heterologous gene expression, and *in vitro* chemical and enzymatic reactions (W. Chen et al., 2017). *Monascus* azaphilone pigments are constructed from integration of two larger biosynthetic building blocks; a non-reduced polyketide and a 3-oxo fatty acid (see Figure 2.4). Labeling experiments using 1-^{13}C-acetate and 2-^{13}C-acetate have shown that both the polyketide, as well as the 3-oxo fatty acid are biosynthesized from one acetyl-CoA unit, and additional malonyl-CoA units (Ogihara, Kato, Oishi, & Fujimoto, 2000). Balakrishnan *et al.* identified the gene *mpPKS5* in *M. purpureus* (Balakrishnan et al., 2013) encoding the PKS catalyzing the formation of the backbone polyketide structure in MP biosynthesis. Furthermore the same research group discovered a gene named *mpp7* responsible for the regioselective attachment of the 3-oxo-fatty acid (Balakrishnan et al., 2014). In 2014, Woo *et al.* (Woo et al., 2014) proposed a biosynthetic pathway after the construction of several knock-out mutants in *Penicillium marneffei*. Their work elucidated that one specific PKS, namely *pks3*, was responsible for most of the biosynthesis. Their proposed pathway, was based on findings by Hajjaj *et al.* who investigated the biosynthetic origin of citrinin in *M. ruber* (Hassan Hajjaj et al., 1999).

One possible biosynthetic pathway (modified from Chen *et al.*) is illustrated in Figure 2.4, on p. 13. The polyketide backbone is made from one acetyl-CoA unit and five malonyl-CoA units, as well as a single methylation at C-9. Formation of the first ring system happens via an aldol condensation of the polyketide backbone structure, linking C-6 and C-11, followed by keto-enol tautomerization, forming the aromatic ring consisting of C-6 to C-11. Following the initial aldol condensation, oxidation introduces a hydroxy group at C-9. This reaction has

been shown to be a result of tailoring activity by the monooxygenase mppF in *M. purpureus* (Bijinu et al., 2014) and the FAD-dependent monooxygenase, MrPigN, in *M. ruber* M7 (Chen *et al.*). Through a reductive release of the polyketide backbone, an aldehyde is formed at C-12, allowing for the subsequent second ring formation between the oxygen atom at C-4, and C-12, to form the heterocyclic isochromene system characteristic for azaphilones. The final step in the biosynthesis is the attachment of the 3-oxo fatty acid to the core bicyclic pyranoquinone. The fatty acid is expected to be synthesized by the fatty acid synthase (FAS) MpFAS2 in *M. purpureus* (Bijinu et al., 2014), and a FAS consisting of the two subunits MrPigJ and MrPigK in *M. ruber* M7. The fatty acid is attached to the C-9-alcohol via a transesterification, followed by an aldol condensation at C-8. In *M. purpureus* the condensation is assisted by the protein Mpp7 in order to control regioselectivity (Balakrishnan et al., 2014), ensuring that the carbon-carbon bond is formed at C-8 rather than at C-10 of the polyketide backbone.

Furthermore, amination of the orange pigments by amine-containing compounds leads to a color shift and the formation of red pigments. This has been proposed to happen via formation of an enamine (Lin et al., 1992). The conversion has been carried out chemically using various amino acids (W. Chen et al., 2017; Lin et al., 1992), but whether the biological conversion is enzyme-mediated or happens entirely spontaneous is still speculative.

It was shown that *T. atroroseus* is missing the classic PKS for MP synthesis. Instead the PKS MitA might be responsible for the production of MP in *T. atroroseus* (Rasmussen, 2015).

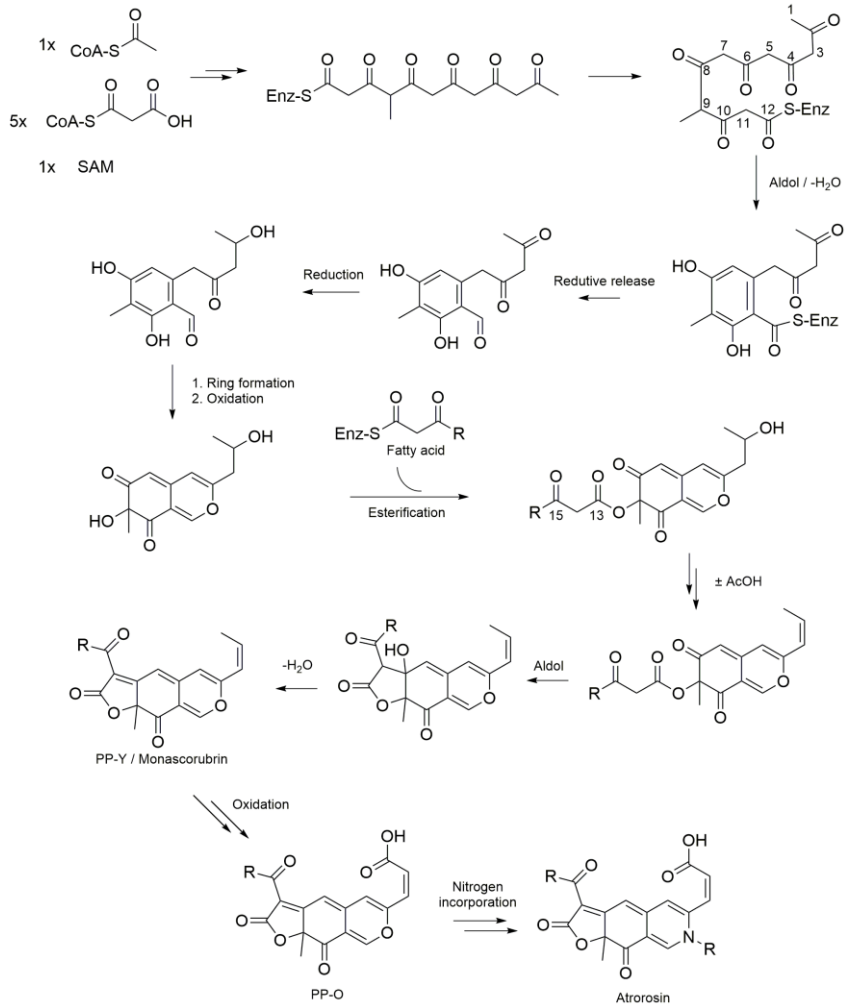


Figure 2.4 Proposed *Monascus* Pigment biosynthesis modified from *Chen* et al. (2017)

2.3.2 Derivatives of *Monascus* Pigments

Several derivatives of the six original MP have been described in the literature. Pigment- derivatives originate by the substitution of the oxygen in monascorubrine or rubropunctatine (described in Figure 2.3) by the nitrogen of the amino group of various compounds such as amino acids, peptides and proteins.

Based on this principle, MP derivatives containing for example glutamate (Blanc et al., 1994; Lin et al., 1992), aspartic acid and alanine (Sato, 1997) have

been identified and characterized. Jung *et al.* produced *Monascus* pigment derivatives by using 20 individual amino acids in submerged cultivation as side chain precursors (Jung et al., 2003). The pigments containing amino acids were found to be more robust towards both temperature and pH changes than the original pigments and demonstrated increased photo stability (Jung et al., 2005). Moreover, new red color hues were exhibited by the newly synthesized pigments (Jung et al., 2003, 2005; Lin et al., 1992). These properties make amino acid derivatives of MP particular interesting for further exploration as food colorants. They can be obtained by either adding the amino acids into the cultivation medium or by chemical synthesis. For the latter, the orange pigments and specific amino acids, or amines are dissolved in ethanol and then mixed (Jo et al., 2014; Xiong et al., 2014).

Not only amino group-containing compounds can be used for the creation of new derivatives. A high glucose concentration of the culture medium has been shown to induce the formation of glucosyl derivatives of the pigments, such as N-glucosylrubropunctamine and N-glucosylmonascorubramine (Hassan Hajjaj et al., 1997). When cultivating *Monascus*, the fungus usually produces a mixture of the six classical MP, and their different derivatives.

Several species among the genus of *Penicillia* have been shown to produce *Monascus*-like azaphilone pigments- most of them without co-producing the mycotoxin citrinin. An international patent was filed in 2007 on production of *Monascus*-like pigments, by the potentially safe strain *Penicillium purpurogenum* (EP 2262862 A2, 2007). In 2013, Frisvad *et al.* re-classified *P. purpurogenum* as *T. atroroseus.* The newly classified species belonging to *Talaromyces*: *T. purpurogenus, T. atroroseus, T. albobiverticillius, T. minioluteus*, and *T. marneffei* have previously been linked in the literature under *Penicillium* species names (Frisvad et al., 2013). This means that some species previously referred to *Penicillium purpurogenum* are now classified as either *Talaromyces atroroseus* or *Talaromyces purpureus.* As a consequence, the species *P. purpurogenum* no longer exists (Frisvad et al., 2013). Confusion regarding species names can therefore still arise, as not all *P. purpurogenum* strains from the literature have been reclassified. In 2013, Frisvad *et al.* reclassified the *P. purpurogenum* IBT 11181 that was used in the patent application as well as several others supposedly *Penicillia* as *T. atroroseus* (Frisvad et al., 2013). Table 2.1 summarizes pigment producing *Penicillium* species that might belong to the species *T. atroroseus*.

Table 2.1 Pigment producing strain *T. atroroseus* represented in the literature

Published fungal name	Reported pigment	Reference
T. atroroseus, classified by Frisvad *et al.*(Frisvad et al., 2013)		
T. atroroseus	Several MP	(Frisvad et al., 2013)
Strain reclassified as *T. atroroseus* by Frisvard *et al.*(Frisvad et al., 2013)		
P. purpurogenum IBT 11181	Monascorubramine	(Mapari, Meyer, Thrane, et al., 2009)
	N- glutarylmonascorubramine	(Mapari, Meyer, Thrane, et al., 2009)
	N- glutarylrubropunctamine	(Mapari, Meyer, Thrane, et al., 2009)
	PP-R	(Mapari et al., 2006)
P. purpurogenum IAM 15392	PP-R (*P. purpurogenum*- red)	(Ogihara et al., 2001)
	PP-V (*P. purpurogenum*- violet)	(Ogihara, Kato, Oishi, Fujimoto, et al., 2000)
	PP-O (*P. purpurogenum*- orange)	(Ogihara & Oishi, 2002)
	PP-Y (*P. purpurogenum*- yellow)	(Ogihara & Oishi, 2002)
P. purporogenum Stoll	Purpuride	(King, 1973)
Strains potentially belonging to *T. atroroseus*, but not examined yet(Frisvad et al., 2013)		
P. purpurogenum DPUA 1275	Yellow, orange, red*	(Santos-Ebinuma et al., 2014; Santos-Ebinuma, Roberto, et al., 2013; Santos-Ebinuma, Teixeira, et al., 2013; Ventura et al., 2013)
P. purpurogenum GH2	Red pigment production*	(Méndez et al., 2011)
Penicillium spp.	Extracellular pigment-production*	(Gunasekaran & Poorniammal, 2008)

***no chemical structure of the pigments were reported**

T. atroroseus is a very promising candidate for MP production, due to its capacity to excrete various different pigments in high yields and its lack of citrinin production (Frisvad et al., 2013; Mapari, Meyer, Thrane, et al., 2009; Ogihara et al., 2001; Ogihara, Kato, Oishi, & Fujimoto, 2000; Ogihara, Kato, Oishi, Fujimoto, et al., 2000). Figure 2.5 shows the chemical structures of MP derivatives associated with *T. atroroseus*. All strains reclassified as *T. atroroseus* will be addressed as *T. atroroseus* for the remainder of this thesis; those, which have not been reclassified officially, will be referred to using the species name used in the respective publications.

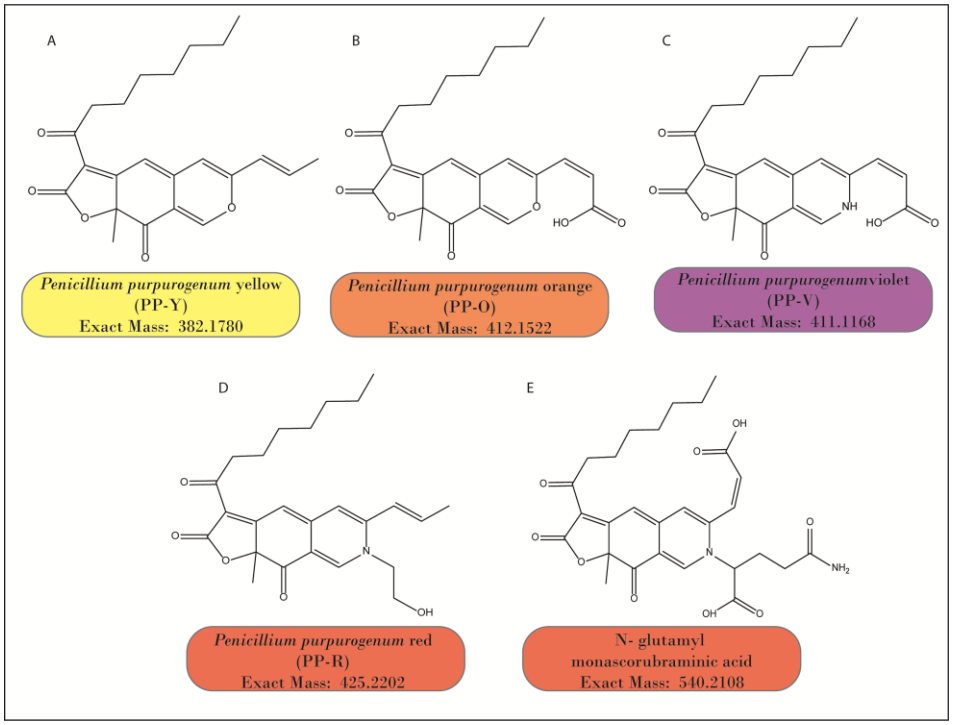


Figure 2.5 Chemical structures and exact masses of MP identified in *T. atroroseus;* *A) Penicillium Purpurogenum*–yellow (PP-Y) (Ogihara et al., 2001), B) *Penicillium Purpurogenum*–Orange (PP-O) (Ogihara & Oishi, 2002), C) *Penicillium Purpurogenum*–Violet (PP-V) (Ogihara, Kato, Oishi, Fujimoto, et al., 2000), D) *Penicillium Purpurogenum*–Red (PP-R) (Ogihara & Oishi, 2002), E) N-glutamyl monascorubraminic acid, isolated from *T. atroroseus* IBT 11181(Rasmussen, 2015).

2.4 From pigment producing fungi to high performance cell factories

For more than 50 years, filamentous fungi have served as industrial cell factories, and large-scale processes based on these organisms are therefore well established. Citric acid production in *Aspergillus niger* is well described and implemented on industrial large scale. Also, the *Aspergillus* genus is one of the favorite expression systems for the production of industrial enzymes (Lubertozzi & Keasling, 2009). Fungal pigment producers include *Blakeslea trispora* which was presented as a new natural source of β-carotene in 1995 (Dufossé et al., 2005) and has been reported to produce up to 44.5 mg per g biomass (Berman et al., 2014; Lampila et al., 1985). It is used in submerged fermentation processes by DSM as a cell factory for food colorants (WO2003038064 A8, 2001; Mapari et al., 2010). *Blakeslea trispora* also produces the carotenoid lycopene (WO2003038064 A8, 2001; López-Nieto et al., 2004). The commercial use of lycopene from *Blakeslea trispora* as a novel food ingredient was approved by the European Parliament in 2006 (Commission Decision 2006/721/EC). Furthermore, the Czech company Ascolor described a process for the production of an anthraquinone type molecule using *Penicillium oxalicum* in 2004, called "Arpink Red"(US 6340586 B1, 2002). However, this process is no longer in production.

One common feature of high performance cell factories is the fact that the strains have been carefully selected. The selected strains were then either highly adapted or engineered to the specific conditions of the process application; or the process conditions were tailored to optimize the production in the strain. The performance of filamentous fungi in lab-scale submerged cultivation determines their suitability for large-scale industrial application and is the result of complex interplay between the physical and chemical parameters of the process and the cellular biology of the fungus (John Villadsen, Jens Nielsen, 2011).

When considering cell factory design of fungal hosts, several challenges exist and are outlined in Figure 2.6. Firstly, non-domesticated species must be screened for production of interesting compounds. This can be done by cultivating the fungus on different agar plates, in shake flasks or by using high throughput screening techniques in micro titer plates. Once a potential candidate is identified, the physiology of the microorganism needs to be described. When talking about natural isolates, where growth rates and morphology in submerged cultures is not known, this step is important for a successful implementation of cultivation in stirred tank reactors. Once the fungus is tested and validated as cultivable, the products of interest (POI) needs to be extracted, identified and quantified. The

establishment of a fungal cell factory is an iterative process coupled to further process design and strain optimization. The challenge here is to balance cellular potential, process design – both up- and downstream, and economic feasibility (Workman et al., 2013).

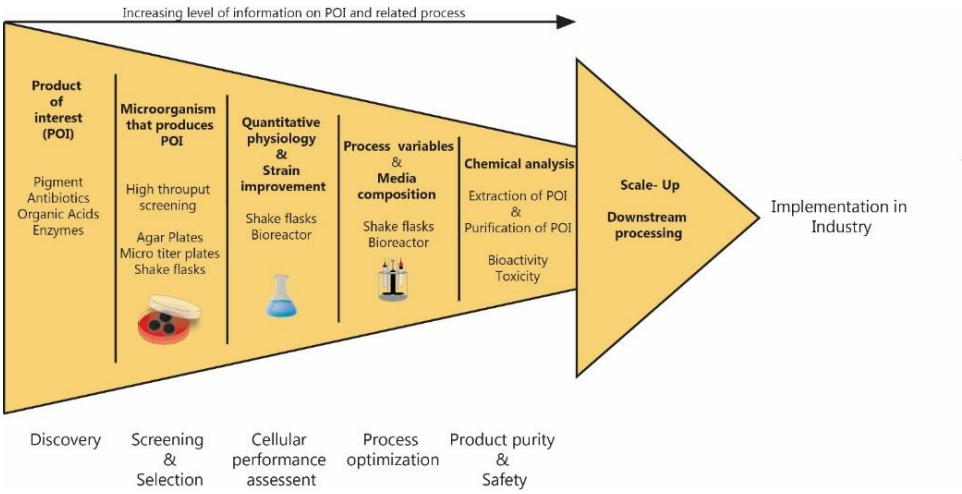


Figure 2.6 Major steps for the establishment of a successful fungal cell factory

2.5 Assessment of the product of interest

In the scope of this Ph.D. thesis the POI refers to "*Monascus* Pigments". A term that usually describes not only one pure pigment but rather to a cocktail of different MP including yellow, orange and red constituents (Woo et al., 2014). The disadvantage of mixtures is that they are difficult to control and monitor because the individual compositions might vary from batch to batch. For identification and especially quantification of potential novel MP, purification strategies are required to separate individual pigments from the mixture. Recovery of pure pigments allows for structural analysis and set-up of quantification tools, such as HPLC methods.

So far no common quantification strategy is available for MP and the published results in terms of pigment yields are difficult to compare across research groups (Table 2.2 on p. 26). In the majority of cases, the absorbance spectrum of the supernatant is measured, and values obtained at specific wavelengths, e.g. at 400 nm, 470 nm or 490 nm are reported (Santos-Ebinuma et al., 2014). Often, there is only limited information regarding the size of the sample volume, the depth of the well or the dilution factor applied in many of the previous studies. Straight forward standard procedures for quantifying individual

pigments from the fermentation broth would therefore be desirable to enable comparison of different processes across studies.

2.6 Fungal cell factories for pigment production

The optimization of cellular performance of a fungal cell factory can be tackled from many different angles. It is an interplay between media constituents, such as carbon sources, nitrogen sources and mineral salts, and culture conditions, e.g. pH, temperature and aeration rate (Babitha et al., 2007a; Mørkeberg et al., 1995; Pedersen et al., 2000; Rajendhran et al., 2002; J. Yang et al., 2015). Figure 2.7 represents the interacting parameters in the process of cell factory design. Once the cell factory together with process and media conditions is identified, quantitative description of the cellular processes in the bioreactor can help to improve the production process even further. To do so, yield coefficients, which are quantitative measures that specify how the cells convert substrates into products (J. Nielsen, 2006), are calculated to assess productivity of the cell factory. Cell factory design usually also includes genetic engineering in order to enhance biosynthesis of the POI.

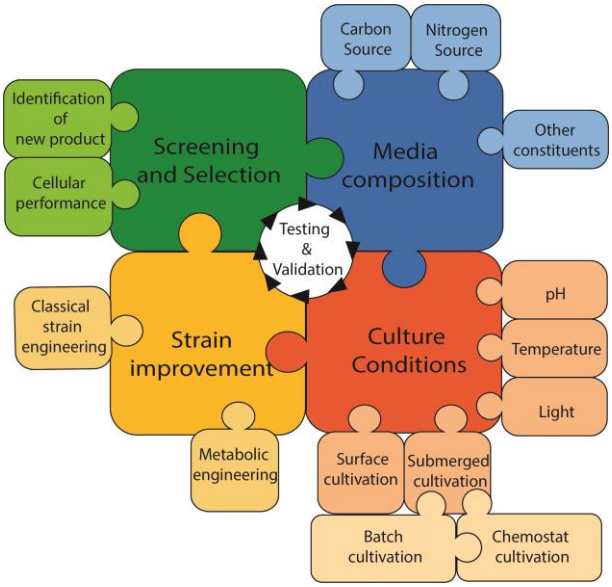


Figure 2.7 Iterative Process of implementing novel cell factories for pigment production

2.6.1 Type of cultivation

2.6.1.1 Solid state versus submerged cultivation

For production of secondary metabolites by filamentous fungi, two cultivation modes can be used: solid-state fermentation (SSF) and submerged fermentation (SmF). SmF involves cultivation in homogeneous aqueous growth medium, while SSF refers to cultures grown on solid surfaces or particles. MP can be produced by both SSF (J C Carvalho et al., 2007; Johns & Stuart, 1991) or SmF (Kang et al., 2014; Yoshimura, 1975) in shake flasks or bioreactors. Products of SSF can directly be used as food colorants (Liu et al., 2010) since both the fungus and its substrate are ground together to a fine colored powder. Products of SmF require extraction before further use.

SSF is a classical process to produce MP in Asia, in which the fungus is inoculated into steamed rice or rice kernels spread on wooden trays and cultured for about 20 days in an air-, moisture-, and temperature-controlled room (Dufossé et al., 2005). As the pigments produced by SSF are unpurified products, their application is limited. SSF also has some limitations in process control, which could be overcome by submerged cultivation (Vendruscolo et al., 2015).

When grown in a submerged cultivation, the fungus can be cultivated in shake flasks or bioreactors using a wide array of defined or complex media. Pigment production processes performed in bioreactors outcompete those performed in shake flasks both in terms of information level and degree of control, but also in terms of better cellular performance. Improved growth and pigment production may be due to better hydrodynamics and oxygen transfer in a stirred tank reactor (Mohamed et al., 2012). When comparing solid state with submerged fermentation, SSF possesses many advantages including simpler production techniques, lower capital investment, lower amount of waste output and easy product recovery (Feng et al., 2012). However, with respect to productivity, cultivation in a stirred tank reactor is more economically viable as it has shorter cultivation times, lower production costs in the long run and higher product quality. It allows up-scaling of the parameters and offers several modes of operation for studying the metabolism, fine-tuning the process and optimizing the production yield (Krairak et al., 2000).

2.6.1.2 Submerged cultivation of filamentous fungi in the bioreactor

The versatility of the bioreactor set-up is expressed in a variety of choice in the operation mode. A bioreactor can be operated in three following modes:

- Batch, where $F_{in}=F_{out}= 0$, *i.e.* the volume in the reactor is constant.
- Continuous, where $F_{in}=F_{out}> 0$, *i.e.* the volume in the reactor is constant.
- Fed-Batch, where $F_{in} > 0$ and $F_{out}= 0$, *i.e.* the volume in the reactor increases.

A batch cultivation is the simplest configuration of all cultivations. It is versatile, because it can be used for many different processes, has low risk of contamination and offers complete conversion of the substrate. One disadvantage of batch cultivation is, that due to the dynamic environment, conditions throughout the experiment change as the substrate is used and products are formed. A typical growth curve for batch fermentation is shown in Figure 2.8.

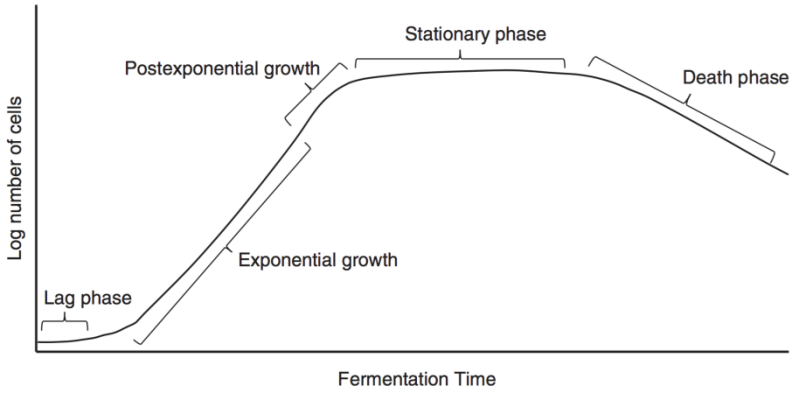


Figure 2.8 Typical phases of batch fermentation

The first phase is the lag phase, where the cells become acclimated to the conditions in the reactor and minimal increase in cell density is observed. Lag time can be reduced or eliminated by selecting an inoculum that has been actively growing in an environment similar to that in the reactor (called pre-culture). After the lag phase, the cells begin propagation. This phase is called the exponential phase with the cells dividing at a constant rate. The growth rates vary greatly among different organisms and reactor conditions. During the exponential growth phase, mostly primary metabolites are produced and the exponential growth may be negatively influenced by substrate or product inhibition. At the end of the exponential growth phase nutrients and substrate diminishes thus the cell growth decelerates and the cells enter a stationary phase where the death rate of the cells equals the rate of growth. In the stationary phase, production of

secondary metabolites occurs. As the nutrients deplete, the death rate exceeds the growth rate and the cells thereby enter the death phase (Wilkins & Atiyeh, 2011).

Continuous cultivations are very attractive from an academic point of view. The concept of steady state offers an excellent opportunity to measure the rate of metabolic reactions or to characterize the morphology of the fungus at a set of well-defined operating conditions (Christensen & Henriksen, 1995), however, in-depth physiological characterization would be required before continuous processes could be designed and implemented for pigment production.

Fed-Batch processes allow very high cell densities and hereby final titers. It is preferable to have studied the organism in continuous cultivation before designing a fed-batch experiment.

T. atroroseus and other related species have so far mainly been studied on agar plates (Frisvad et al., 2013; Mapari et al., 2006) or in submerged cultivation using shake flasks (Méndez et al., 2011; Santos-Ebinuma et al., 2014; Ventura et al., 2013). No bioreactor data is available yet, so there are still unexplored opportunities for studying these potential cell factories by using different submerged cultivation strategies in a bioreactor based set-ups.

2.6.2 Assessment of classical physiological parameters

Key cellular performance indicators, most importantly growth rate, utilization of the substrate and yield of desired products can be used to evaluate a given cell factory. Different cultivation modes and conditions can be used to produce and study various physiological states. For a reliable quantitative physiological characterization, it is paramount that the cultivations are performed in a controlled environment and that the reproducibility can be demonstrated. Such studies provide data on which process optimization can be based, but also give an insight into the active metabolic pathways based on the nutritional requirements and measured products (Workman et al., 2013).

Furthermore, rates of substrate consumption, product formation and biomass accumulation can be determined during a cultivation process by measuring their concentrations in the medium. The substrate uptake rate and product formation rate are volumetric rates and given in $(\frac{m}{V \cdot t})$. However, often it is convenient to normalize the rates to make them comparable between fermentation experiments. They are referred to as specific rates (r_i, $(\frac{m}{m_{DW} \cdot t})$.One example is the specific growth rate μ_{max} (J. Nielsen, 2006). The growth rate is often the key parameter

used when screening potential cell factories. A fast growing fungus is better suited for industrial processes because it lowers the production length and thereby production costs and the risk of contamination. Information on biomass accumulation in pigment producing *Penicillium* species or *Talaromyces* species is incomplete and so far growth rates in bioreactors have not been reported for any of those strains. Growth rates for *Monascus* species have previously been investigated and vary from 0.02 h^{-1} for *Monascus* spp. (Krairak et al., 2000) to 0.04 h^{-1} for *Monascus ruber* (M. Chen & Johns, 1993; Hassan Hajjaj et al., 1997; Said et al., 2014).

Other important design parameters are yield coefficients. These constants help express the amount of substrate funneled into biomass and metabolic products. They are given in ratios of the specific rates. The yield for substrate conversion into biomass is:

Eq. 1.1 $$Y_{SX} = \frac{\mu max}{r_s}$$ Yield of biomass per substrate

Similarly the yield for substrate conversion into product is:

Eq. 1.2 $$Y_{SP} = \frac{r_p}{r_s}$$ Yield of product per substrate

Yield coefficients who refer to the substrate, describe how the substrate is distributed among the different products. It is also possible to calculate yield coefficients with biomass of the microorganism as reference point. This yield coefficient describes product formation in ratio to biomass.

Eq. 1.3 $$Y_{XP} = \frac{r_p}{\mu max}$$ Yield of product per biomass

Yield coefficients allow comparison of different media recipes. For example, if an organism is able to grow fast and accumulates a lot of biomass (high Y_{SX}), but is only able to produce very low amounts of the desired product (low Y_{SP}) that particular media is not promoting the production of the POI very well. All yields can be calculated in g/g, mole/ mole or for carbon containing compounds c-mole/c-mole (John Villadsen, Jens Nielsen, 2011). It is also possible to calculate a yield coefficient based on nitrogen with the unit nitrogen-mole (n-mole/ n-mole).

2.6.3 Media composition

In order to ensure growth of the fungus and formation of the POI, certain nutritional requirements have to be met. Nutritional requirements for growth of filamentous fungi are water, sources of energy, carbon, nitrogen, mineral salts and oxygen (Stanbury et al., 2017). The carbon source is typically a carbohydrate. Glucose is a very common carbon source, but also sucrose or lactose or mixtures thereof can be used. The nitrogen source can either be organic (e.g. peptone, amino acids) or inorganic (e.g. KNO_3, $(NH_4)_2SO_4$). Sources of mineral salts ensure supply of phosphate, sulfate and metals, needed for important metabolic functions. All these factors should be investigated in order to find an optimal media recipe that guarantees maximal pigment yields. Media compositions can contain minimal or complex ingredients. Complex media are often cheaper because they contain waste products from the agriculture industry like molasses, corn steep liquor or yeast extract. They consist of complex mixtures of many different compounds ensuring supply of carbon and nitrogen sources as well as minerals. A fundamental problem with complex media is however that their composition can vary a from batch to batch and with the season, might contain unknown impurities and this may affect product and biomass yields and reproducibility of the fermentation process. The use of a complex medium might also hinder product recovery and downstream processing. This is why defined media are better suited for academic purposes because all quantities of the ingredients are known. An equation describing stoichiometry for growth and product formation can be found in Eq. 1.4:

Eq. 1.4 Carbon source + nitrogen source + O_2+ other requirements → biomass+ products+CO_2+ H_2O+ heat

The profile of produced metabolites can be tailored by media design. In particular the production of secondary metabolites is energetically costly and is subject to general physiological control that responds to environmental factors. Therefore their production can be influenced by the choice of carbon and nitrogen sources or the addition of specific inducers (Martin & Demain, 1980; Vining, 1990; J.-H. Yu & Keller, 2005). Glucose, usually an excellent carbon source for growth, often interferes with the formation of secondary metabolites. Also many secondary metabolic pathways are negatively affected by nitrogen sources favorable for growth, e.g., ammonium salts. As a result, a slowly assimilated nitrogen source like an amino acid or a nitrate salt is often used as the nitrogen source to encourage high production of secondary metabolites (Adrio & Demain, 2003).

2.6.4 Cultivation parameters

In addition to the media composition, different cultivation parameters can be used to improve cellular performance. Cultivation parameters include incubation time as well as size and type of inoculum (Santos-Ebinuma, Teixeira, et al., 2013), or can involve physical parameters such as pH, temperature, oxygen supply, light or simply the cultivation mode. The reactor and impeller design can also influence product formation (Mohamed et al., 2012). Many of these parameters have already been studied for *Monascus* species and from these results conclusions could be drawn to optimal cultivation conditions for *Talaromyces* related species. Table 2.2 summarizes reported growth conditions and pigment yields for *T. atroroseus* and other related species.

2.7 Strain improvement

Metabolic engineering is the practice of optimizing genetic and regulatory processes within the cell to increase the cells' production of a certain substance. But the lack of genetic tools available for most fungal species has for many years been the major obstacle for exploring the molecular biology and biochemistry of all but a few model fungi. One potential tool for genetic engineering of filamentous fungi was presented recently. The CRISPR-Cas9 based system is now adapted for use in filamentous fungi. The system is simple and versatile, as RNA guided mutagenesis can be achieved by transforming a target fungus with a single plasmid (Nødvig et al., 2015).

In the past decade, great progress has been made in studying and manipulating *Monascus* spp. at the genetic level using molecular biology techniques to restrain the citrinin production or increase the yields of pigments and other beneficial products such as monacolin K. Until now, hundreds of papers about *Monascus* molecular biology have been published (Shao et al., 2014). The complete genome of *Monascus purpureus* YY-1 was provided in 2015 (Y. Yang et al., 2015), consisting of eight chromosomes and 7491 genes. The genome from *Monascus ruber* is also publicly available (He et al., 2013).

Table 2.2 Reported optimal process conditions for *T. atroroseus* and other related species

Fungus	Optimal process parameters	Assessment of color	Max Color yield	Ref.
P. purpurogenum GH2	pH 5, 24 °C, 260 h, 200 rpm, 15 g/L xylose, 3 g/L $NaNO_3$	Absorbance measurement of supernatant at 500 nm. Yields were calculated as the ratio of the amount of pigment produced (in nm) at a certain time to the biomass generated in the same time.	2.4 g/L [a]	(Méndez et al., 2011)
Penicillium spp.	pH 9, 30 °C, 4 days, 200 rpm, soluble starch, peptone	Absorbance measurement of supernatant at 530 nm with spectrophotometer.	UA530=1238 [b]	(Gunasekaran & Poorniammal, 2008)
P. purpurogenum DPUA 1275	pH 6.5, 30 °C, 360 h, 150 rpm, 30 g/L sucrose, 5 g/L yeast extract, 5 mycelia discs	Absorbance measurement of SN at 400, 470, and 490 nm with spectrophotometer.	UA400=3.08 [b] UA470=1.44 [a] UA490=2.27 [b]	(Santos-Ebinuma, Teixeira, et al., 2013)
	pH 4.5, 30 °C, 336 h, 150 rpm, 50 g/L sucrose, 10 g/L yeast extract		UA400=3.10 [b] UA470=2.50 [b] UA490=2.04 [b]	(Santos-Ebinuma et al., 2014)
T. atroroseus IBT11181	pH 5, 30 °C, 2 days, 20 g soluble starch, 3 g NH_4NO_3, 2 g yeast extract	Detection by thin layer chromatography, identification of PP-V and PP-R by H^1 NMR and C^{13} NMR		(Ogihara, Kato, Oishi, Fujimoto, et al., 2000; Ogihara & Oishi, 2002)
	pH 5, 30 °C, 4 days, 200 rpm, 20 g soluble starch, 2 g yeast extract	Detection by thin layer chromatography, identification of PP-Y and PP-O by H^1 NMR and C^{13} NMR		(Ogihara & Oishi, 2002)
	Plates: pH 6.5, 25 °C, 7 days (CYA/YE) Shake flasks: pH 6.5, 25 °C, 7 days, 150 rpm (CZ)	Absorbance measurement of SN, prior adjustment to 0.40±0.04 at their respective absorption maxima with purified water. Determination of CIELAB color coordinates using a Chromameter.		(Mapari et al., 2006)

a. Yield is not quantitative even though given in g/L but refers to absorbance measurements per biomass
b. Absorbance given as Units of Absorbance at a given wavelength in nm.

Among *Talaromyces* genomes *T. stipitatus* (Davison et al., 2012; Joardar et al., 2012) and *T. marneffei* (Woo et al., 2010) are publicly available, however *T. stipitatus* has never been reported to produce MP, and *T. marneffei* is an opportunistic human pathogen. Therefore, these two are not suitable choices for MP gene cluster studies. In a study in 2013, 454 sequences of *T. atroroseus* IAM15392 (Published as *P. purpurogenum*) were used as basis for qPCR expression comparison of genes (glnA: glutamine synthetase; gdhA: glutamate dehydrogenase) involved in the ammonia assimilation pathway in response to media concentration of L-glutamate and L-glutamine (Arai et al., 2013). In 2017, the genome of *T. atroroseus* IBT11181 was made publicly available (Thrane et al., 2017) and by the use of CRISPR-Cas9, already a new gene responsible for the production of polyketide-nonribosomal peptide hybrid products was recently identified in *T. atroroseus* (M. L. Nielsen et al., 2017).

The recently published genome of *T. atroroseus* can now pave the way for omics-driven analyses, which are an essential approach to the evaluation of genetic regulation of an organism because they offer a holistic view of cellular functions. Furthermore, it would allow the development of metabolic engineering strategies in order to manipulate *T. atroroseus* to enhance production of fungal pigments.

2.8 Pigment properties

With the purpose of being used as a food colorant, MP and their derivatives need to fulfill very specific requirements. Heat, light and pH stability are desirable characteristics as well as solubility in water. But it is equally important to demonstrate the safety of the pigments and to rule out a potential toxicity. Only few reports on toxicity of MP can be found, probably due to his extensive historical use. Furthermore their bioactivity needs to be evaluated carefully to properly declare potential additional features of the pigment. As there are so many derivatives of the MP, every modification of the molecule, for example the incorporation of an amino acid, a carboxylic acid or a glycosyl-group might lead to slightly changed biological and chemical properties (Wong & Koehler, 1983).

The beneficial properties of the *Monascus* product "red rice" are commonly known in Asia for centuries and also more recent studies demonstrate their useful biological activities for medical purposes (Bianchi, 2005; Gheith et al., 2008; Hsu & Pan, 2014; Mohan Kumari et al., 2009; Patakova, 2013; C. C. Yu et al., 2008). Health beneficial properties of MP include antimicrobial, antifungal, antiviral, antioxidant, cytotoxic and anti-inflammatory activities as well as anti-mutagenic and anti-cancer properties or even potential anti-obesity characteristics (Akihisa

et al., 2005; Feng et al., 2012; Jang et al., 2014; Osmanova et al., 2010). The rather non-selective bioactivity of azaphilones is due to the formation of vinylogous γ-pyridones (Osmanova et al., 2010); a reaction, in which the oxygen atom in the pyrane ring is exchanged for nitrogen from amino group-containing compounds. This modification explains also why most of the bioactive properties are associated with orange and yellow pigments, and not with red ones. These qualities should be further investigated in order to assess their potential impact for functional food or drug discovery.

Since *T. atroroseus* and other related species produce the same group of pigments, similar properties are likely to be associated with them. But so far, no bioactivity tests have been performed on the pigments exclusively linked to *T. atroroseus*. Unfortunately, the majority of pigment analysis in the literature is based on absorbance measurements, and therefore the exact structures and compositions of the produced pigments remains unknown. Identification and purification of the individual pigments is a requirement to determine their detailed properties.

3 Materials & Methods

This chapter presents procedures and protocols that were used during the experimental work of this thesis. Most of the experimental procedure stayed the same across all chapters. This includes propagation of *T. atroroseus*, set-up of bioreactors, sample procedures and chemical analysis of the red pigments. Additionally, each chapter contains a small experimental section with procedures exclusively relevant for the individual chapter

3.1 Strain and propagation

The strain used in this study was *Talaromyces atroroseus* IBT 11181 (DTU strain collection). *T. atroroseus* spores were propagated on CYA agar plates and incubated at 30 °C for 7 days. Spores were harvested with 0.9% sodium chloride solution (NaCl), filtered through mira-cloth, centrifuged and then re-suspended in 0.9% NaCl solution. The spore concentration was determined by using a Burker-Turk counting chamber. All cultivations were inoculated to give an initial spore concentration of 10^6 spores/mL.

3.2 Reagents

All purchased solvents and reagents were acquired from Sigma-Aldrich (St. Louis, Missouri, USA), ultra-pure water was made with a Milli-Q system (Millipore, Burlington, Massachusetts, USA).

3.3 Trace metal solution

The trace metal solution consisted of $CuSO_4 \cdot 5\ H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.04 g/L), $FeSO_4 \cdot 7\ H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2\ H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7\ H_2O$ (8 g/L).

3.4 Propagation in shake flasks

Cultivations were performed in 500 mL shake flasks with a working volume of 100 mL. The flasks were incubated at 150 rpm at the temperatures indicated in the different chapters.

3.5 Bioreactor based batch cultivations

All reactor based cultivations were carried out in Sartorius 0.5 L, 1 L or 2 L bioreactors (Satorius, Stedim Biotech, Goettingen, Germany) and were used as indicated in the corresponding sections. Reactors were equipped with 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer's standard procedures. The bioreactor was sparged with sterile atmospheric air and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific, Waltham, MA/USA), calibrated monthly with gas mixtures containing 5% (v/v) CO_2, 0.04 % (v/v) ethanol and methanol, 1% (v/v)

argon, 5% (v/v) and 15% (v/v) oxygen all with nitrogen as carrier gas (Linde Gas, AGA, Enköping, Sweden). The pH was controlled by automatic addition of 2 M NaOH and H_2SO_4. Temperature settings for the individual cultivations are given in the respective chapter. During the germination phase, both stirring and aeration were low to ensure that hydrophobic spores were not expelled from the medium. Aeration started with 0.1 vvm and initial stirring was 100 rpm. The start conditions were slowly changed to reach final process conditions after 24 h (800 rpm, 1 vvm).

T. atroroseus in batch cultivation was cultivated until carbon depletion and the culture had entered stationary phase as monitored by off-gas CO_2- concentration. Samples for HPLC, LC-MS, and absorbance analysis were taken across the entire cultivation for quantitative physiology analysis.

3.6 Bioreactor based chemostat cultivation

Chemostat cultivations were performed in Sartorious 1 L bioreactors with an equivalent working volume. The reactors were equipped with 2 Rushton six-blade disc turbines. Chemostat cultivation were initiated and cultivated as batch until late exponential phase, when feed medium supplementation was initiated. The feed medium was identical to the batch medium. Feed supply and bioreactor outflow were controlled by a gravimetrically controlled peristatlic pump ensuring constant bioreactor volume. The dilution rate of the chemostat was $D= 0.07\ h^{-1}$. The cultivation was performed at 30 °C, 800 rpm, 1 vvm.

3.7 Sampling

Samples for dry weight (DW) analysis, HPLC, absorbance analysis, and LC-MS analysis were taken regularly throughout the cultivations. Samples intended for HPLC, absorbance, and LC-MS were filtered through a sterile cellulose acetate filter (Frisenette, Knebel, Denmark) with a pore size of 0.45 µm in order to separate biomass from the filtrate. The samples were frozen and kept at -20 °C until analysis.

3.8 Dry weight analysis

Dry weight (DW) was assessed on filters which were pre-dried in a microwave for 20 min, kept in a desiccator for a minimum of 10 min and weighed. For DW analysis, the filters were placed in a vacuum filtration pump and ca. 10 mL of culture broth was added. Subsequently the filters with the biomass were dried in

a microwave for 20 min and kept in a desiccator for a minimum of 10 min before being re-weighed. The weight of the biomass was determined as the difference of the filter weight before and after sample application.

3.9 Analysis of extracellular metabolites by HPLC

Glucose, sucrose and fructose were detected and quantified using an Agilent 1100 HPLC system equipped with a refractive index and Diode array detector (Agilent Technologies, Waldbronn, Germany) and with an Aminex HPX-87H cation-exchange column (BioRad, Hercules, Ca, USA). Compounds were separated by isocratic elution at 30 °C, with 5 mM H_2SO_4 at a flow rate of 0.8 mL min-1. Quantification was performed using a six-level external calibration curve with glucose detected at a wavelength of 210 nm and sucrose and fructose, by refractive index measurements.

3.10 Quantitative physiological analysis

Maximum specific growth rates (μ_{max}) were estimated from exponential fits of biomass concentrations determined gravimetrically as a function of time. For the cultivation in 0.5 L bioreactors, μ_{max} calculation were based on accumulated CO_2 production. The correlation between DW and accumulated CO_2 is illustrated in Figure 3.1. This correlation allows for the calculation of certain yield coefficients to be based on accumulated CO_2-values rather than on DW.

Overall yield coefficients (Y_{SX}, Y_{SP}, Y_{XP}) for batch cultivations were calculated with the final values of product and biomass based on the initial concentration of carbon source. Yields are given in c-mole/c-mole, if not other indicated.

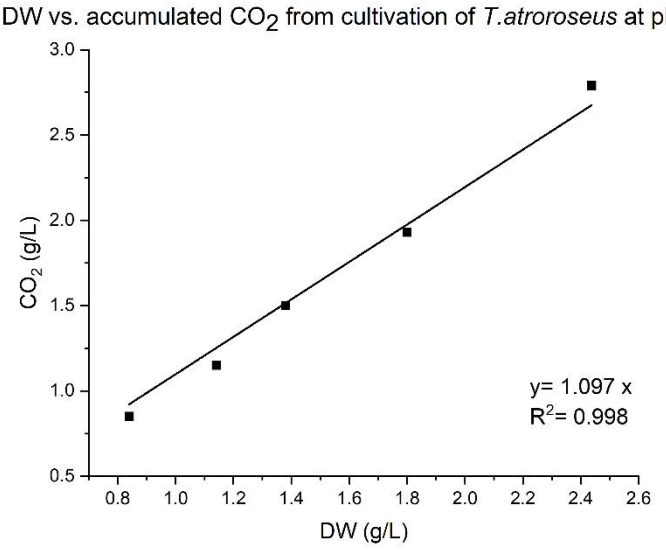


Figure 3.1 Correlation of DW measurements (g/L) and accumulated CO_2 (g/L) of submerged cultivation of *T. atroroseus* at pH 5.

3.10.1 Overall CO_2- production in batch cultivation

Yield of CO_2 on sucrose was determined for batch cultivations. First, exhaust CO_2-concentrations (g/L) were calculated and are shown in Eq. 2.1.

Eq. 3.1 $$\sum_1^{i=n} \frac{(Q_i + Q_{i-1}) \cdot (t_i - t_{i-1})}{2V_R} \cdot M_{CO_2}$$ (accumulated CO_2 at time t_n,g/L)

Where t_n is the sample time (h), V_r is the volume of the bioreactor (L), M_{CO2} is the molecular mass of CO_2, (44.0095 g/mol) and Qi is defined as mol CO_2 produced per hour at a given time point. Q_i is calculated using Eq. 2.2.

Eq. 3.2 $$Q_i = F_{air,i} \cdot \frac{(\%\, CO_{2_{ti}} - \%\, CO_{2_{t0}})}{V_m \cdot 100}$$ (CO_2 production rate, g/L)

Where $F_{air,i}$ is the flow of air (L/h) at the given time point, $\%CO_{2,ti}$ is the CO_2 percentage in the off gas air from the bioreactor at the given time point and $\%CO_{2t0}$ is the background concentration of CO_2 in the gas flow. V_m is the molar volume of CO_2 and derived from the ideal gas low shown in Eq.2.3.

Eq. 3.3 $$V_m = \frac{V}{n} = \frac{R \cdot T}{P}$$ (law of ideal gases)

Where R is the ideal gas constant (0.08314 $\frac{J}{mol \cdot K}$), T is the temperature in the off-gas analyzer (298.5 K) and P is the ambient pressure (0.9885 bar).

3.11 Chemical analysis of the pigments

Most of the chemical analysis was performed by Ph.D. student Thomas Isbrandt.

3.11.1 Extraction and purification of atrorosins

Filtered and centrifuged fermentation broth was extracted three times, with 1/3 volume of EtOAc, at pH 3 (adjusted with formic acid). The combined EtOAc phases were evaporated to 100 mL and extracted twice with water (1:1) at pH 8 (adjusted with ammonium hydroxide). The water phase was readjusted to pH 3 with FA and extracted two time with EtOAc, followed by evaporation, to yield >95% pure pigment (a mixture of atrorosins and N-amino acid monascorubramine, ratio>10:1 by LC-DAD-MS).

The final purification of atrorosins was performed on a Gilson 332 semi-prep HPLC system equipped with a Gilson 172 diode array detector, using a LUNA II C18 column (250 mm x 10 mm, 5 μm, Phenomenex), with a water/acetonitrile gradient with 50 ppm trifluoroacetic acid (TFA).

3.11.2 Extraction and purification of PP-O isomers

For purification of the two PP-O isomers, the fermentation liquid was extracted two times with EtOAc to give 1.2 grams of crude extract which was loaded onto an Isolera One (Biotage, Uppsala, Sweden) automated flash system equipped with a diol column and eluted stepwise with dichloromethane (DCM), DCM:EtOAc (1:1), EtOAc, EtOAc:MeOH (1:1), and MeOH. The enriched orange fraction (0.9 g) was subjected to solid phase extraction (SPE) to remove co-eluting impurities. The diol fraction was dissolved in MeOH and loaded onto an equilibrated Oasis WAX solid phase extraction (SPE) column (Waters, Milford, Massachusetts, USA) and washed with 3 column volumes (CVs) of MeOH followed by elution of the orange pigment with 3 CVs of 2% TFA in MeOH. The orange fraction, practically only containing a mixture of *cis*- and *trans*- PP-O, was purified on the same Gilson semi-prep HPLC system as the atrorosins, but using a water/MeOH gradient with 50 ppm TFA and a Kinetex Core-Shell C18 column (250 mm x 10 mm, 5 μm), in order to separate the two isomers.

Measuring of optical rotation of all isolated compounds was done on a Perkin-Elmer 341 Polarimeter (Perkin Elmer, Waltham, Massachusetts, USA) using a 10 cm cell.

3.11.3 Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS)

Analysis was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 μm) with a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and remaining for 3 min (0.35 mL/min, 60 °C). An injection volume of 1 μL was used. MS detection was performed in positive detection mode on an Agilent 6545 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 L/min, sheath gas temperature of 300 °C and flow of 12 L/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Mass spectra were recorded at 10, 20 and 40 eV as centroid data for m/z 85–1700 in MS mode and m/z 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Lock mass solution in 70:30 methanol:water was infused in the second sprayer using an extra LC pump at a flow of 15 μL/min using a 1:100 splitter. The solution contained 1 μM tributylamine (Sigma-Aldrich) and 10 μM Hexakis (2,2,3,3-tetrafluoropropoxy) phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The $[M + H]^+$ ions (m/z 186.2216 and 922.0098 respectively) of both compounds were used.

3.11.4 1D and 2D NMR analysis

Spectra were recorded on a Bruker Avance 800 MHz spectrometer (Bruker, Billerica, MA, USA) located at the Department of Chemistry at the Technical University of Denmark. NMR spectra were acquired using standard pulse sequences. The solvent used was either DMSO-*d*$_6$ or CD_3OD, which were also used as references with signals at δ_H = 2.50 ppm and δ_C = 39.5 ppm for DMSO-*d*$_6$ and δ_H = 3.31 ppm and δ_C = 49.0 ppm for CD_3OD. Data processing and analysis was done using TopSpin 3.5pl7 (Bruker). *J*-couplings are reported in hertz (Hz) and chemical shifts in ppm (δ). For all natural pigments, 1D proton, edHSQC, and HMBC were recorded, and for atrorosin S, LR-HSQMBC, 1,n-ADEQUATE, and 1,1-ADEQUATE were also measured. Compounds were

measured in CD_3OD, except atrorosin Q which was measured in DMSO-d_6. For the semi-synthesised nature identical pigments, only 1D proton and edHSQC were recorded.

3.12 Quantitative analysis of the red pigments

The absorbance values of the individual pigment solutions were determined using a Synergy 2 photo spectrum (BioTek, Germany) and a 96 well microtiter plate. 150 µL of sample broth of each amino-acid-pigment-solution were scanned in the range of 200-700 nm and maximum absorbance values were determined. Absorbance at 500 nm indicated presence of red pigments.

After purification a standard curve was generated for Atrorosin-E, as well as for a mixture of the two PP-O isomers. Both standard curves are shown in Figure 3.2. The standard curve of atrorosin-E was used for determination of the concentration of red pigments in the fermentation broth and the standard curve of PP-O was used for determination of the total content of orange compounds respectively. The equations for calculation of orange (PP-O) and red (atrorosin-E) pigments can be found in Eq. 3.4 and Eq. 3.5.

Eq. 3.4 $Abs_{480\ nm} = 8.12 \cdot conc_{PP\text{-}O}\ (g/L)$ Concentration (total PP-O):

Eq. 3.5 $Abs_{500\ nm} = 10.17 \cdot conc_{atr\text{-}E}\ (g/L)$ Concentration (atrorosin-E):

All pigment concentration values presented in this thesis before purification of atrorosin-E, have been calculated retrospectively from measured absorbance values.

3.13 Proof of absence of citrinin

All LC-MS samples were compared to a standard of citrinin ($m/z = 251.0290$) to rule out citrinin production by *T. atroroseus*. No citrinin production was detected in any of the extracts from *T. atroroseus*.

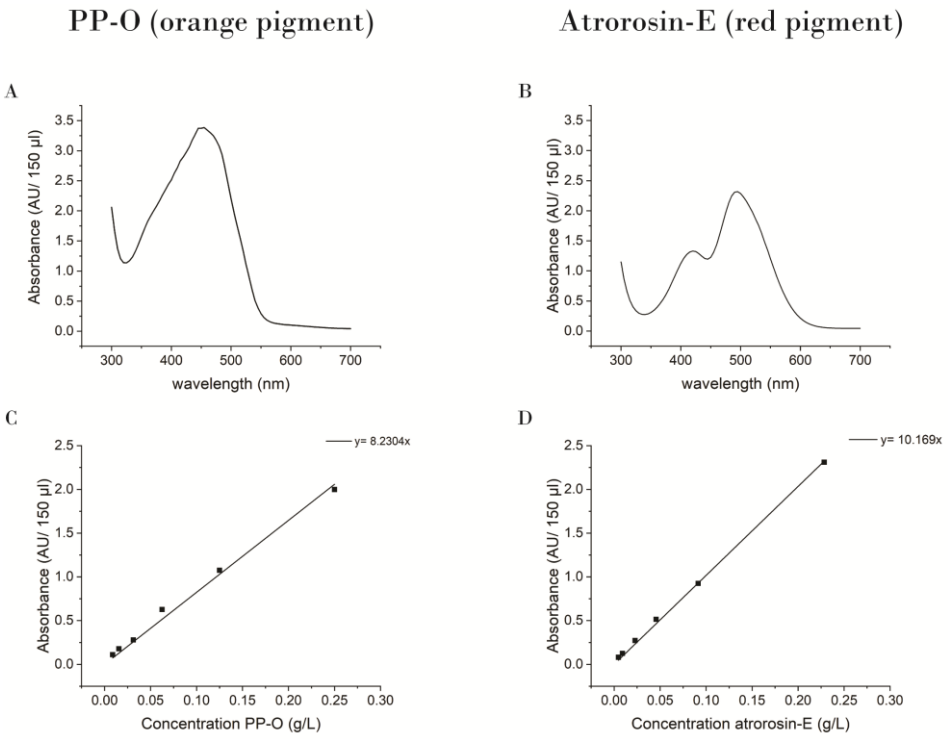


Figure 3.2 A) Absorbance spectrum from PP-O. B) Absorbance spectrum from atrorosin-E. C) Standard curve for quantification of PP-O. D) Standard curve for quantification of atrorosin-E

4 Design of a cultivation medium supporting pigment production in *T. atroroseus*

This chapter presents the results from screening experiments for a medium recipe promoting high pigment yields in the strain *Talaromyces atroroseus IBT11181*. For that, different media components were investigated, and the most promising recipe was validated in bioreactors. The major focus here was on carbon and nitrogen sources because they heavily influence the secondary metabolic profile of filamentous fungi (Scervino et al., 2011).

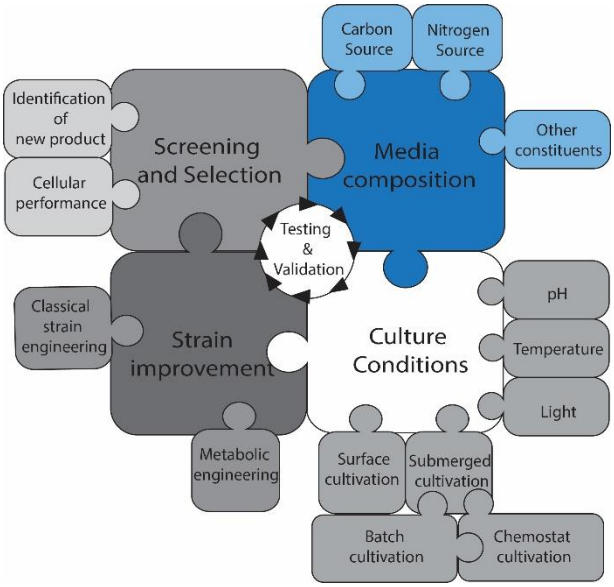


Figure 4.1 Iterative Process of implementing novel cell factories for pigment production; Media design is highlighted

4.1 Media design for triggering pigment production in *T. atroroseus*

Secondary metabolite production in fungi is dependent on external stimuli, and can be promoted or repressed by careful media design (Brakhage, 1998). Media design represents thus an important tool in the toolbox of cell factory design for MP production (Figure 4.1) and many studies have been performed in order to find the optimal media recipe in *Monascus* spp. (Koehler, 1980; Lin & Demain, 1991; Said et al., 2014). In *Monascus ruber* it is reported that pigment production is growth associated (Said et al., 2014), but in the MP producing species *P. purpurogenum GH2* and *T. atroroseus*, however, it appeared that red pigment production occurs at the expense of biomass production (Mapari, 2008; Méndez et al., 2011). But, at present this statement is only speculative as titers rather than yields have been reported in the literature. Final biomass values were not put in relation to the amount of carbon source used and are thus difficult to compare.

When designing a cultivation medium the following criteria should be met (Stanbury et al., 2017):

1. High growth rate

2. Maximum product yield per substrate
3. Maximum rate of product formation
4. Minimum yield of undesired side product formation
5. Minimal problems in media preparation and sterilization
6. Minimal problems in product recovery

For the purpose of studying a specific process, a minimal medium is preferred over a complex medium. This is because, a minimal medium assures consistent quality and all individual ingredients are known and accountable. The use of minimal media also eases product recovery (John Villadsen, Jens Nielsen, 2011). For the evaluation of different media the following criteria will be applied:

1. Is *T. atroroseus* able to grow on the media? This was evaluated by assessing accumulated biomass, calculating the growth rate(μ_{max}) and yield coefficients.
2. Does the *T. atroroseus* produce the desired red pigments? This was assessed by chemical analysis such as LC-MS and by absorbance measurement with a spectometer.
3. How are the yields of the pigment compared to accumulated biomass or carbon used in the process? Yield coefficients such as Y_{SP} and Y_{XP} can be calculated and compared. Y_{SP} is a measure of pigment production in relation to the use of carbon and Y_{XP} refers to pigment production in relation to biomass.

4.1.1 Selection of carbon source

The carbon source (CS) serves as substrate and is converted into biomass, CO_2 and other metabolic products, and in the case of *Talaromyces atroroseus* into azaphilone pigments. Different CS can be used more or less effectively to supply the nutritional and energetically demands of the cell. Microorganisms can utilize a wide range of different CS. Depending on the circumstances, they express a subset of different hydrolytic enzymes to exploit the given CS (Stanbury et al., 2017).

CS for pigment production in submerged cultivation of *T. atroroseus* and related species include soluble starch (Ogihara, Kato, Oishi, Fujimoto, et al., 2000), potato starch (Mapari, Meyer, Thrane, et al., 2009), sucrose (Santos-Ebinuma et al., 2014), glucose (Ogihara, Kato, Oishi, Fujimoto, et al., 2000), fructose (Ogihara, Kato, Oishi, Fujimoto, et al., 2000), galactose (Ogihara, Kato, Oishi, Fujimoto, et al., 2000), mannose (Ogihara, Kato, Oishi, Fujimoto, et al., 2000), arabinose (Ogihara, Kato, Oishi, Fujimoto, et al., 2000) and xylose (Méndez et al., 2011). Gunasekaran *et al.* screened the effect of 11 CS on pigment

production and concluded that soluble starch promoted the highest pigment yields, followed by maltose and glucose (Gunasekaran & Poorniammal, 2008). However, a study on *P. purpurogenum (*old name for *T. atroroseus*) showed that sucrose was the most promising CS in terms of pigment production (Santos-Ebinuma, Teixeira, et al., 2013). *P. purpurogenum* was also shown to grow on cellulose, wheat straw and wheat bran, but no pigment production was documented (Steiner et al., 1994).

4.1.2 Selection of nitrogen source

Inorganic compounds such as ammonium chloride or ammonium nitrate, and organic nitrogen like yeast extract, monosodium glutamate (MSG) and peptone, have been reported to be nitrogen sources (NS) for both growth and pigment production in submerged grown *Monascus* spp. (Feng et al., 2012). However, studies suggest, that if organic NS are used, the addition of free amino acids to the culture medium can further enhance pigment production (Patakova, 2013). But not all amino acids seem to promote pigment production equally well and particularly leucine was associated with an even negative effect (Lin & Demain, 1994). Supplementing the cultivation medium with amino acids was also shown to improve red pigment production when inorganic NS were used. Higher yields were reported in *Monascus* compared to when the inorganic NS was used alone (Yoshimura, 1975). The increased yields are probably caused by incorporation of the amino acid into azaphilone core, instead of using it for growth and propagation (Lin et al., 1992). When amino acids were added as supplements, the corresponding MP derivatives with the amino acid moiety, e.g. N-glycinemonascorubramine or N-glutarylrubropunctamine, were identified in the MP mixture (Jung et al., 2003, 2005; C. Kim et al., 2006).

MSG is also a commonly used NS. The nitrogen atom from glutamate is converted into free ammonium by oxidative deamination. This reaction is catalyzed by glutamate dehydrogenase (Berg et al., 2006). The use of glutamate as a NS also obviates the need for its own synthesis and can directly, or after conversion to glutamine, be used for biosynthesis (Said et al., 2014).

When looking at inorganic NS, filamentous fungi generally prefer ammonium as NS over nitrate because nitrate must be reduced in an energy demanding process before it is accessible for anabolic processes (M. Chen & Johns, 1993)

For *Monascus*, reduced biomass but increased pigment formation was reported when inorganic NS, such as ammonium chloride, sodium nitrate and ammonium nitrate (Carels & Shepherd, 1977) were used. Nitrates limit growth but stimulate spore and red pigment formation (Carels & Shepherd, 1977). The use of

ammonium nitrate as the NS has been found to result in formation of mainly cell-bound orange pigments (Lin & Demain, 1995).

Pigment production with *T. atroroseus* is reported on both, complex NS such as yeast extract (Mapari, Meyer, Thrane, et al., 2009; Ogihara & Oishi, 2002) and on inorganic NS *e.g.* ammonium nitrate (Arai et al., 2012, 2013; Ogihara, Kato, Oishi, Fujimoto, et al., 2000; Ogihara & Oishi, 2002). The latter together with yeast extract promotes PP-V (*P. purpurogenum*- violet) and PP-R (*P. purpurogenum*- red) production, but when yeast extract alone is used as NS, PP-O (*P. purpurogenum*- orange) and PP-Y (*P. purpurogenum*- yellow) are produced (Ogihara & Oishi, 2002). The inorganic NS ammonium and nitrate can both be used for PP-V production, but ammonium has been found to result in higher pigment yields than nitrate (Arai et al., 2012).

It seems thus possible in MP producing species to selectively produce different color components (yellow, orange or red pigments) through nitrogen source selection (Shi et al., 2015).

4.1.3 Other medium components

Co-factors such as metal ions and salts greatly affects fungal metabolism. Minerals like magnesium sulfate, potassium chloride, and phosphate are considered macronutrients and have shown to influence pigment production in *Monascus* (Lin & Demain, 1993). Lin *et al.* reported that high concentrations of both phosphate (above 70 mM) and magnesium sulfate (above 16 mM) have an inhibitory effect on cell growth and pigment production (Lin & Demain, 1991). In contrast, potassium chloride concentration was found not to affect cell growth or pigment production significantly (Lin & Demain, 1991). The negative effects of high concentrations of phosphate and magnesium are likely be caused by an inhibition of pigment synthase action (Lin & Demain, 1993). The positive effects of trace metals, especially Zn^{2+} have been shown to be due to stimulation of growth and enzyme action. Until this thesis work, no studies on the effect of minerals on *T. atroroseus* or related species have been performed.

4.2 Experimental strategy

For the design of a pigment production medium, different carbon and nitrogen sources were screened for their effect on pigment production in submerged cultivation of *T. atroroseus*. As starting point for the media design strategy served three different medium compositions presented by Sameer Mapari

in his Ph.D. thesis "Chemotaxonomic Exploration of Fungal Biodiversity for Polyketide Natural Food Colorants" in 2008 (N1, N11 and CYA) (Mapari, 2008). Since, *T. atroroseus* is a newly classified species, not much more literature was found on cultivation condition of this species. N1 is a minimal medium whereas N11 and CYA also contain complex ingredients. In a second screening step, four different CS, namely glucose, sucrose, xylose, and glycerol were tested in order to understand more about the carbon metabolism. As a third step different inorganic nitrogen sources were investigated, followed by a last step where different concentrations of magnesium sulfate were examined to assess a possible inhibitory effect on pigment production. All screening experiments were performed in shake flasks. Finally, KNO_3, $(NH_4)_2SO_4$, and NH_4NO_3 were studied in 2 L bioreactors at constant pH. The general medium design strategy can be found in Figure 4.2.

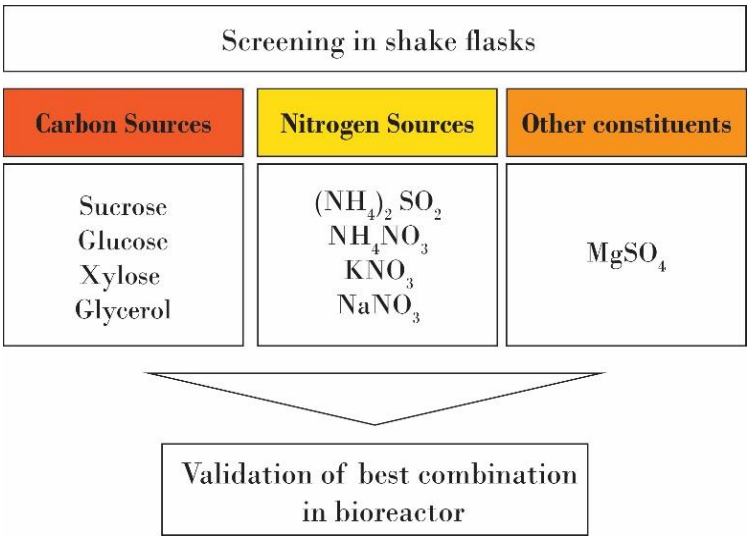


Figure 4.2 Experimental strategy for medium design

4.3 Materials & Methods

A detailed description of spore preparation, propagation of the fungus in submerged cultivation and sampling can be found in chapter 2.

Shake flask cultivation were carried out at 25 °C, 150 rpm and an initial pH of 5 was adjusted with 2 M NaOH and 2 M HCl. Shake flask experiments had a working volume of 100 ml.

Bioreactor experiments were carried out in Sartorius 2L bioreactors with equivalent working volume at 25 °C. The pH was maintained at 5 by automatic addition of 2 M NaOH and H_2SO_4.

Media recipes were the following:
N1 (modified from Mapari): Glucose (20 g/L), KNO_3 (4 g/L), KH_2PO_4 (3.2 g/L), KCl (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and 1 ml/L of trace metal solution (Mapari, 2008).

N11 (modified from Mapari): Glucose (1 g/L), Lactose (20 g/L), Yeast extract (18.2 g/L), Potato Starch (10 g/L), Corn steep liquor (5.6 g/L), KCl (1.8 g/L), $FeSO_4 \cdot 7H_2O$ (0.04 g/L), $MgSO_4 \cdot 7H_2O$ (1.8 g/L), NH_4NO_3 (7.3 g/L) and 1 ml/L of trace metal solutions (Mapari, 2008).

CYA: Czapek dox broth (35 g/L), Yeast extract (5 g/L)

Czapek dox broth: Sucrose (30 g/L), $NaNO_3$ (3 g/L), KH_2PO_4 (1 g/L), KCl (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L) $FeSO_4 \cdot 7\ H_2O$ (0.01 g/L),

Trace metal solution: $CuSO_4 \cdot 5\ H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.04 g/L), $FeSO_4 \cdot 7\ H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2\ H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7\ H_2O$ (8 g/L).

Carbon Screening: Medium N1 was used. The following carbon sources were screened: Glucose, Sucrose, Xylose and Glycerol. All CS were added to a concentration of 20 g/L.

Nitrogen Screening: Medium N1 was used with 20 g/L sucrose as carbon source. The following nitrogen sources were screened: KNO_3, $(NH_4)_2SO_4$, $NaNO_3$, NH_4NO_3. All NS were added to a concentration of 0.1 nitrogen-mol/L.

$MgSO_4$ Screening: Medium N1 was used with 20 g/L sucrose as carbon source and 10 g/L KNO_3 as nitrogen source. $MgSO_4 \cdot 7H_2O$ was added in the concentration (g/L): 0.1, 0.25, 0.75, 1, 2, 5, 10.

Pigment yield was determined by measuring the absorbance at 500 nm and converting the value into g/L with the help of the standard curve from atrorosin-E shown in Figure 3.2 on p.37.

4.4 Results and Discussion

4.4.1 Complex versus minimal media

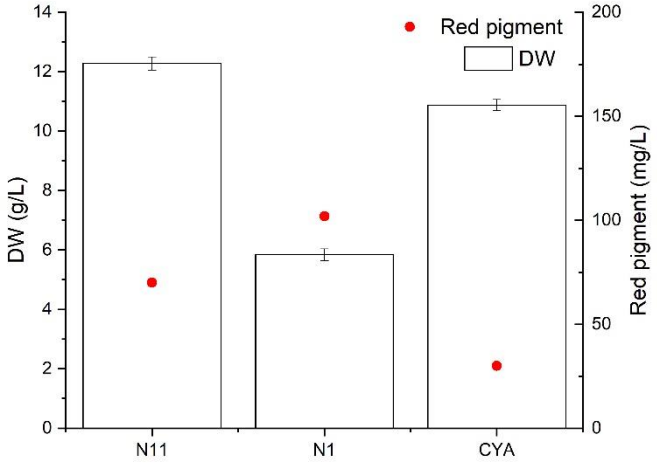


Figure 4.3 Biomass accumulation and pigment production in g/L in three different medium compositions (N1: minimal medium, N11, CYA: complex media). Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5.

In the first screening step, three media originally described by Sameer Mapari were tested. These include two complex media (N11 and CYA) and one minimal medium (N1). They were evaluated in terms of biomass accumulation and pigment production. Figure 4.3 illustrates that pigment production was best on the minimal medium N1. Biomass accumulation was however significantly higher in both complex medium compositions. The reason for that was probably because complex ingredients like yeast extract, potato starch or corn steep liquor are more easily assimilated by the fungus. Assimilation of inorganic NS and oligosaccharides is slower and requires a lot of energy, but favors secondary metabolite production. This could explain the low biomass values and the increase pigment production on N1. These observations were in accordance with results obtained in *Monascus*, reporting that complex medium ingredients can stimulate fungal growth, but suppressed pigment production (Patakova, 2013).

A relevant tool for evaluation of different media compositions is the yield coefficient Y_{SP}, which expresses the carbon source /product ratio and is an indication for the effectivity of carbon source to product conversion of a process.

Since, N11 and CYA both are complex media, determination of the exact amount of initial carbon is difficult. Therefore, the yield coefficient Y_{XP} was calculated and is presented in Table 4.1. Y_{XP} is a measure of product formation per biomass.

Table 4.1 Yield coefficients Y_{XP} of *T. atroroseus* on N11, N1 and CYA. Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5.

Media	N11	N1	CYA
Y_{XP} (c-mole pigment/c-mole biomass)	0.006	0.026	0.009

Pigment production in *T. atroroseus* in the minimal medium N1 was with a Y_{XP} of 0.026 c-mole/c-mole, 3 times higher than in the complex media N11 and CYA. As a result, N1 served as the starting point for the subsequent screening of carbon and nitrogen sources.

4.4.2 Screening of carbon sources

Four carbon sources were screened for their influence on the cellular performance of *T. atroroseus*. The screened carbon sources were sucrose (C12-sugar), glucose (C6- sugar), xylose (C5- sugar) and glycerol (C3- polyol). Shake flask cultivation with *T. atroroseus* were successfully conducted with sucrose, glucose, and xylose as CS and a photograph of the shake flask cultivation is shown in Figure 4.4.

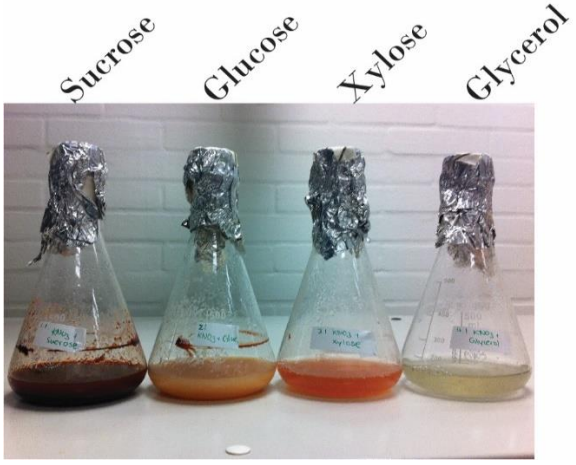


Figure 4.4 From left to right: sucrose, glucose, xylose and glycerol with KNO_3 as nitrogen source. Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5.

Visually, sucrose yielded the highest pigment titers. Neither growth nor pigment production was observed with glycerol as carbon source. Yield coefficients (Y_{SX}, Y_{SP}, Y_{XP}) were calculated and are presented in Table 4.2.

Yield coefficients confirmed the visual impression from the photograph, that sucrose was the superior carbon source for pigment production in *T. atroroseus* under the tested conditions. Both product related yields, Y_{SP} and Y_{XP}, were highest on sucrose with 0.027±0.001 c-mole/ c-mole and 0.038±0.005 c-mole/c-mole respectively. *T. atroroseus* grew well on glucose and decent on xylose, but pigment production was significantly decreased.

Table 4.2 Yield coefficients from carbon screening in *T. atroroseus*. Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5 with KNO_3 as nitrogen source.

	$\mathbf{Y_{SX}}$ $(\frac{c-mole}{c-mole})$	$\mathbf{Y_{SP}}$ $(\frac{c-mole}{c-mole})$	$\mathbf{Y_{XP}}$ $(\frac{c-mole}{c-mole})$
Sucrose	0.405±0.021	0.027±0.001	0.038±0.005
Glucose	0.422±0.001	0.009±0.001	0.018±0.002
Xylose	0.221±0.015	0.005±0.001	0.020±0.003
Glycerol	no growth on glycerol		

Assimilation of glucose is fast and easy for *T. atroroseus* and the highest biomass values were thus reached on glucose with an Y_{SX} of 0.422±0.001 c-mole/c-mole. However, pigment production in *T. atroroseus* could be subject to glucose repression explaining the lower pigment yields on glucose as CS. These findings are in accordance with Santos-Ebinuma *et al.* (Santos-Ebinuma, Teixeira, et al., 2013). Glucose repression is quite common for secondary metabolites in filamentous fungi and has also been reported in *Monascus* (M. Chen & Johns, 1993). Sucrose is a good CS to avoid carbon repression as it enables slow enzymatic release of glucose and fructose. Sucrose is a C12-sugar and can still be easily assimilated by *T. atroroseus*, leading to rather fast biomass accumulation. Xylose might be more difficult to use for *T. atroroseus* as xylose is a C4-sugar and *T. atroroseus* set of hydrolytic enzymes might not be as highly expressed as for C12 and C6 sugars. The use of xylose as a CS therefore resulted in lower biomass accumulation. The results on glycerol suggested that *T. atroroseus* lacks the enzymatic capability for C3-catabolism as neither growth nor pigment production was observed on glycerol.

The results from the carbon screening indicated that there might not be a positive correlation between cellular performance in terms of growth and in terms of pigment production as Y_{SX} was highest on glucose, but the highest Y_{SP} was reached on sucrose.

4.4.3 Screening of nitrogen source on sucrose

From the comparison of N1, CYA and N11, it could be concluded that minimal constituents are preferred for pigment production in *T. atroroseus* over complex ones. For this reason, only inorganic nitrogen sources were investigated in the nitrogen screening and the results are presented in Table 4.3.

The hypothesis from the carbon screening stating a negative correlation of pigment production and biomass accumulation was further strengthened by screening for the best nitrogen source. It revealed a clear trade-off between biomass accumulation and pigment production. Ammonium based nitrogen sources favored growth, as higher biomass values were reached on $(NH_4)_2SO_4$ and NH_4NO_3 (Table 4.3).

Table 4.3 Yield coefficients from nitrogen screening in *T. atroroseus*. All yields are given in c-mole/ c-mole. Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5 with Sucrose as carbon source.

	Y_{SX} $(\frac{c-mole}{c-mole})$	Y_{SP} $(\frac{c-mole}{c-mole})$	Y_{XP} $(\frac{c-mole}{c-mole})$	Pigment yield (g/L)	Observed color hue
KNO_3	0.332±0.004	0.010±0.001	0.029±0.001	0.14±0.008	Red
$NaNO_3$	0.382±0.020	0.003±0.001	0.007±0.001	0.04±0.001	Red
$(NH_4)_2SO_4$	0.444±0.002	0.002±0.001	0.004±0.001	0.03±0.001	Orange
NH_4NO_3	0.487±0.002	0.002±0.001	0.003±0.001	0.02±0.001	Orange

Both KNO_3 and $NaNO_3$ did not support growth of the fungus to the same extend. In regards to pigment production the best results were reached on a nitrate containing nitrogen source. KNO_3, yielded 0.14 g/L of pigment. Pigment yields on $NaNO_3$ were 3,5-fold lower than on KNO_3. The difference in pigment production between KNO_3 and $NaNO_3$ can only be explained by a potential influence of potassium or sodium. High concentrations of sodium have been reported to decrease pigment production in *Monascus* (Tseng et al., 2000). Surprisingly, on KNO_3 and $NaNO_3$, the excreted pigment was red, whereas on $(NH_4)_2SO_4$ and NH_4NO_3 the secreted pigment appeared orange (see Figure 4.5).

The results from the nitrogen screening should be interpreted together with understanding the different nitrogen assimilation mechanisms of filamentous fungi.

Nitrate is imported into the cells by nitrate transporters where it is then reduced to nitrite and ammonium by nitrate and nitrite reductase (Arai et al., 2012). This requires the *de novo* synthesis of these two enzymes, a process which depends on both nitrogen de-repression and specific induction by nitrate (Marzluf, 1997). Nitrate is thus a slowly assimilated nitrogen source. Arai *et al.* demonstrated active nitrate assimilation in *T. atroroseus* and hypothesized further that for the nitrogen incorporation into the pyranoid ring of the pigment, the nitrate available in the culture medium is used (Arai et al., 2012).

Ammonium on the other hand is taken into the cell and is then converted to glutamine and glutamate through an ammonium assimilation process (Arai et al., 2013). This is a fast step promoting fast growth. No reduction is required. Ammonium, in even low concentrations, can suppresses nitrate assimilation when both sources of nitrogen are present together (Morton & MacMillan, 1953), explaining why results of the cultivation on NH_4NO_3 are similar to those from $(NH_4)_2SO_4$.

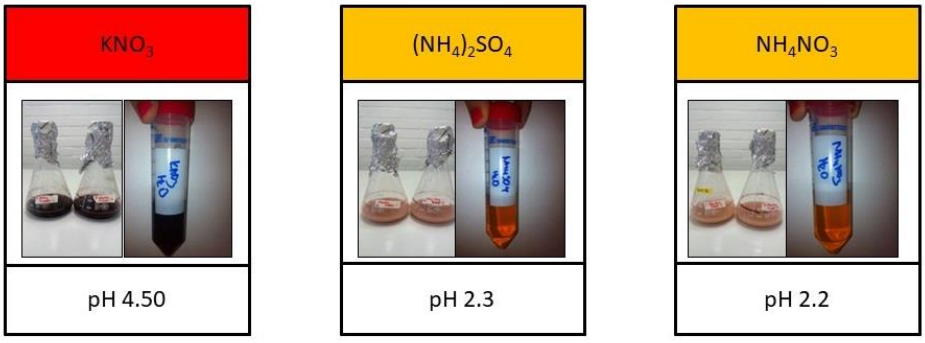


Figure 4.5 Final pH of SN from cultures with KNO_3, $(NH_4)_2SO_4$, and NH_4NO_3. Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5.

Cultivation with KNO_3, $(NH_4)_2SO_4$, and NH_4NO_3 resulted not only in very different pigment profiles but also in significant differences in the final pH value of the three cultivation. Final pH values are illustrated in Figure 4.5. The cultivation on $(NH_4)_2SO_4$, and NH_4NO_3 both had an orange supernatant (SN) and a final pH of 2.3. This was because the use of ammonium lead to acidification of the cultivation medium (Morton & MacMillan, 1953). The fungus took up

NH_4^+, incorporated it into proteins as NH_2 and rejected the protons into the medium (Said et al., 2014).

The cultivation containing KNO_3 had a clear red SN and a final pH of 4.5. During nitrate consumption, the pH of the culture medium rose as nitrate was reduced to ammonium. The different pigment profiles of KNO_3, $(NH_4)_2SO_4$, and NH_4NO_3 could potentially be explained with the measured differences in the pH of the cultivation medium. pH control in shake flasks is difficult and pH can only be buffered or simply monitored.

Observations of different colored pigments on nitrate and ammonium are in accordance with Carels and Shepherd, who reported red pigment formation at a high pH of 6.5 and nitrate as nitrogen source, and orange pigments formation at a pH of 2.5 and ammonium as nitrogen source (Carels & Shepherd, 1977).

This could indicate, that the pH of the cultivation medium affected pigment production in *T. atroroseus* and that pH control of the cultivation process is strongly recommended (Carels & Shepherd, 1978; M. Chen & Johns, 1993; Shi et al., 2015).

4.4.4 Other constituents including magnesium sulfate

The final step in the media screening was the investigation of constituents such as magnesium sulfate, monopotassium phosphate, sodium chloride, potassium chloride and different concentration of the trace metal solution. Only varying concentrations of magnesium sulfate showed to have an impact on pigment production in *T. atroroseus* and thus only these results are presented here. In order to compare the results with the other media compositions Y_{XP} was calculated and the relation between $MgSO_4$-concentration and pigment production is illustrated in Figure 4.6.

Plotting Y_{XP} as a function of the $MgSO_4$-concentration showed a steep increase of Y_{XP} with increasing concentrations of $MgSO_4$. Y_{XP} peaked with 0.061 ± 0.004 c-mole/c-mole at a $MgSO_4$-concentration around 2 g/L and decreased thereafter. This is in accordance with the literature reporting that $MgSO_4$ is required for pigment production but that high concentrations thereof act inhibitory (Lin & Demain, 1991, 1993). Lin and Demain (1991) stated an optimal $MgSO_4$ concentration of 3.8 g/L in *Monascus* and hypothesize that the negative effects of high concentrations of $MgSO_4$ were due to inhibition of pigment synthase action.

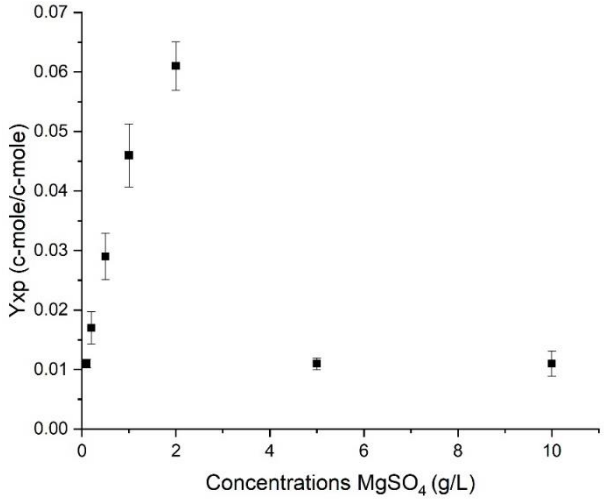


Figure 4.6 Y_{XP} in relation to $MgSO_4$ concentration of the medium. Cultivation were performed in shake flasks at 25 °C, 150 rpm at a starting pH of 5.

4.4.5 *T. atroroseus* pigment production medium

As a summary from the previous experiments, a suitable medium recipe for pigment production in submerged cultivation of *T. atroroseus* was proposed and can be found in Table 4.4. Screening experiments with the filamentous fungus *T. atroroseus* in shake flasks were not always successful, due to limited process control. Data from experiments investigating different concentrations of KH_2PO_4, KNO_3 and trace metal solution were not shown due to inconclusive results and lack of reproducibility. The pH of the medium seemed to affect pigment production, but could not be tightly controlled in a shake flasks set-up. Limited mixing and gas transfer could also negatively influence reproducibility when cultivating filamentous fungi in shake flasks.

However, those findings did partly influence the final medium compositions. The concentration of KNO_3 was adjusted to 10 g/L, and also the concentrations of KH_2PO_4, KCl and the trace metal solution were increased. CaCl and NaCl were added additionally like seen for submerged cultivation media of *Aspergillus niger* (Poulsen et al., 2012).

Table 4.4 *T. atroroseus* pigment production medium. N1 served as the starting point for medium design.

Ingredients g/L	N1	*Talaromyces atroroseus* medium
Glucose	20.0	1
Sucrose		20.0
KNO_3	4.0	10
KH_2PO_4	3.2	10
KCl	0.2	0.5
$MgSO_4 \cdot 7H_2O$	0.2	2
CaCl		0.1
NaCl		1
Trace metals (ml)	1.0	2

4.4.6 Batch cultivation on KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3 at pH 5

In order to validate the proposed pigment production medium, bioreactor experiments were conducted. To investigate whether the choice of nitrogen source or the pH caused the difference in color profile in the shake flasks experiments, *T. atroroseus* was cultivated using KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$ at a constant pH of 5. Three exemplary cultivation plots, illustrating biomass accumulation, sucrose consumption, CO_2-production and pigment production are shown in Figure 4.7- 4.9 and key cultivation parameters are summarized in Table 4.5.

Table 4.5 Different nitrogen sources in batch experiment at constant pH 5.

	μ_{max} (h^{-1})	Pigment yield (g/L)	Y_{SX} ($\frac{c-mole}{c-mole}$)	Y_{SC} ($\frac{c-mole}{c-mole}$)	Y_{SP} ($\frac{c-mole}{c-mole}$)	Y_{XP} ($\frac{c-mole}{c-mole}$)
KNO_3	0.061 ±0.002	0.365 ±0.040	0.334 ±0.001	0.481 ±0.020	0.012 ±0.001	0.081 ±0.009

NH_4NO_3	0.064 ±0.009	0.161 ±0.084	0.422 ±0.007	0.463 ±0.019	0.005 ±0.002	0.027 ±0.014
$(NH_4)_2SO_4$	0.070 ±0.003	0.198 ±0.064	0.456 ±0.001	0.606 ±0.013	0.008 ±0.002	0.040 ±0.012

Surprisingly, at the selected pH of 5 all three nitrogen sources supported red pigment production in *T. atroroseus*, unlike what was observed in shake flasks. Just as in the shake flask experiment, KNO_3 yielded the highest pigment yields with 0.365±0.040 g/L and the product related yield coefficients, Y_{SP} and Y_{XP}, are thus highest on KNO_3. In regards to biomass accumulation, nitrate performed the poorest with a Y_{SX} of only 0.334±0.04 c-mole/c-mole (Table 4.5).

The cultivation with KNO_3 had a lag phase of around 40 h. DW accumulation followed an almost linear path resulting in a rather low growth rates of only 0.06 h^{-1}. CO_2- production increased first exponentially, but already after 55 h, exponential CO_2- production ceased and only a minor increase was detected afterwards. After 125 h, CO_2- exhaust dropped indicating the end of the cultivation. Carbon depletion was reached already after 75 h, but pigment production and DW accumulation continued until the drop of CO_2.

On NH_4NO_3 the same tendencies were observed as on KNO_3. Neither CO_2, nor DW increased exponentially throughout the entire cultivation, but the CO_2-exhaust plateau was shorter compared to KNO_3. Pigment yields were with 0.161± 0.084 g/L lower than on KNO_3, but also here pigment production continued after carbon depletion was reached.

Also on $(NH_4)_2SO_4$ exponential increase of CO_2 was only observed in the beginning. However, a slightly higher growth rate μmax of 0.07 h^{-1} could be calculated based on DW measurements. CO_2- production remained almost steady from 70 to 110 h, like seen on KNO_3 and NH_4NO_3. It peeked at 125 h and then dropped at the same time carbon depletion was reached. Pigment production however continued until 150 h and reached a maximum of 0.198± 0.064 g/L.

Overall, on $(NH_4)_2SO_4$, more CO_2 was produced then on both nitrate containing nitrogen sources, suggesting a less stressed metabolism (Y_{SC} in Table 4.5). Y_{SX} was also highest on $(NH_4)_2SO_4$ followed by NH_4NO_3. KNO_3 had a significant lower Y_{SX}. This could be due to the problematic of nitrate assimilation as discussed earlier. It also appeared that on NH_4NO_3 and KNO_3 growth continued after depletion of sucrose suggesting that some carbon-storing intermediate was produced.

Contrary to the shake flasks experiments, cultivation of *T. atroroseus* in bioreactors revealed similar cellular performance on all three nitrogen sources including red pigment production.

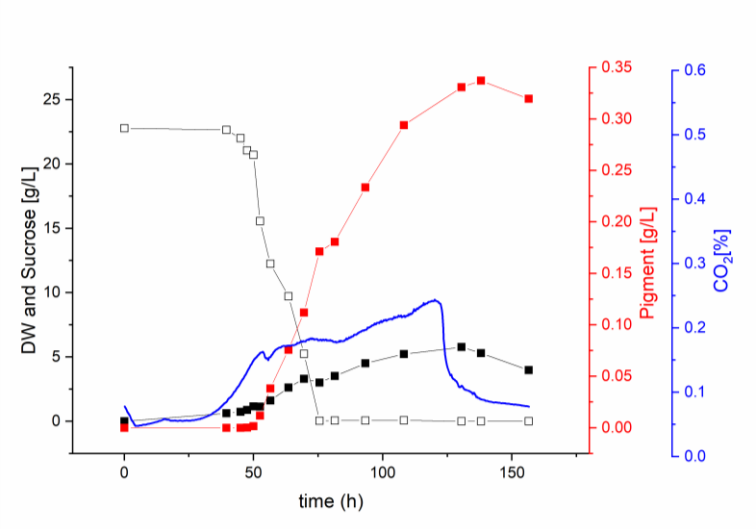


Figure 4.7 Cultivation curve of *T. atroroseus* on KNO_3. Bioreactor experiments were performed in 2 L reactors at 25 °C, 800 rpm, 1 vvm and at a pH of 5.

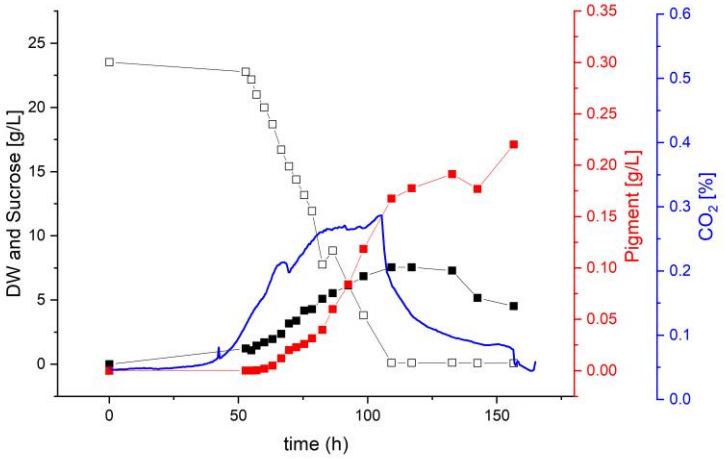


Figure 4.8 Cultivation curve of *T. atroroseus* on NH_4NO_3. Bioreactor experiments were performed in 2 L reactors at 25 °C, 800 rpm, 1 vvm and at a pH of 5.

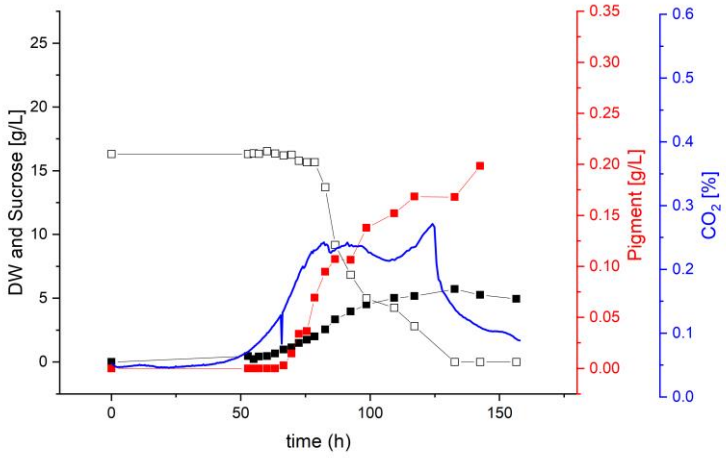


Figure 4.9 Cultivation curve of *T. atroroseus* on $(NH_4)_2SO_4$. Bioreactor experiments were performed in 2 L reactors at 25 °C, 800 rpm, 1 vvm and at a pH of 5.

4.4.6.1 Color profile of KNO_3 and $(NH_4)_2SO_4$

The fermentation broths of the cultivation with KNO_3 and $(NH_4)_2SO_4$ were analyzed further to determine the pigment composition in the fermentation broth. In the case of KNO_3, pigment production started with the formation of orange pigments and as the cultivation continued, the fermentation broth changed color hue towards red (Figure 4.10 B). The orange pigments had an absorbance maximum around 480 nm (Figure 4.10 A). LC- MS analysis could identify PP-O (m/z 413.1595) as the orange component in the early samples. The final fermentation broth had two local maxima at 400 and 500 nm and contained a mixture of red unknown compounds. No "classic" *Monascus* Pigments could be detected in the fermentation broth of *T. atroroseus* when cultivated on KNO_3.

On $(NH_4)_2SO_4$, no production of PP-O was observed in the early samples, but slightly pink pigments were detected in the fermentation broth (Figure 4.10 D). They also changed their color hue towards red later in the cultivation. An analysis revealed the presence of PP-V (m/z 412.1755) in the early samples. The final fermentation broth contained both, PP-V and a mixture of unknown red pigments. Both PP-V and the pigment mixture had two absorbance maxima at 400 and 500 nm (Figure 4.10 C). The identification of PP-V is in accordance with Ogihara *et al.* (2001), who claimed that ammonium containing media would lead to the production of PP-V in *T. atroroseus* (Ogihara & Oishi, 2002).

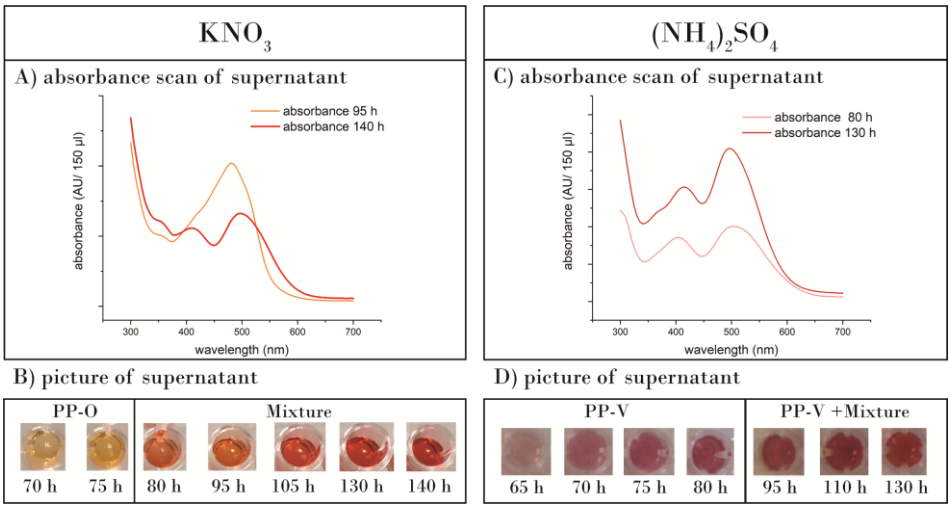


Figure 4.10 Color development of SN in submerged cultivation of *T. atroroseus* on KNO_3 and $(NH_4)_2SO_4$.

The results from the bioreactor experiments indicated that pigment production in *T. atroroseus* is greatly influenced by both the nitrogen source selection and the pH of the culture medium. Whereas pH seemed to influence if red or orange pigments are produced., the nitrogen source seemed to affect which kind of red pigment was produced by the fungus. Because of these findings, studies of different pH values will be addressed in the following chapter to investigate if the pH of the culture medium acts like an turn-on/ turn-off switch for red pigment production in *T. atroroseus*.

4.5 Conclusion

For high yielding cell factories, the design of a suitable fermentation medium is the first step towards the successful implementation of a biotechnological process.

In this study a suitable pigment production medium for *T. atroroseus* was proposed, featuring sucrose as carbon source and KNO_3 as nitrogen source. During the screening process, the pigment yield per biomass (Y_{XP}), was increased from 0.026 c-mole/ c-mole to 0.045 c-mole/c-mole by altering different media constituents and their concentrations. By moving the experiments from the dynamic and uncontrolled environment of shake flasks into the controlled environment of a bioreactor, Y_{XP} was further increased to 0.08 c-mole/c-mole. This was potentially due to pH control and better aeration conditions. Furthermore, different pigment color hues were detected on KNO_3 and

$(NH_4)_2SO_4$, speculated to be caused by different nitrogen assimilation mechanisms. No red pigment production could be achieved on $(NH_4)_2SO_4$ and NH_4NO_3 in shake flasks without pH control, but both nitrogen sources promoted red pigment production in the bioreactor at a pH value of 5 indicating a superior role of the culture pH on pigment production in *T. atroroseus*.

This study also demonstrated that yields coefficients like Y_{XP} allowed comparison of different media recipes and showed to be excellent tools for medium evaluation.

5 Designing a cultivation process for enhanced pigment production in *T. atroroseus*

The aim of this chapter is to describe process conditions favoring red pigment production in *T. atroroseus*. Different cultivation methods are compared to determine their influence on cellular performance of *T. atroroseus*. A suitable cultivation temperature was determined along with a suitable pH. Finally, knowledge from the pH screening was combined in a batch cultivation set-up containing a pH-switch.

The experiments presented in the chemostat section were conducted by M.Sc. student Anne Sofie Simonsen and can be found in her Master's Thesis "Design and Control of Pigment Production Processes using the Filamentous Fungus *Talaromyces atroroseus*".

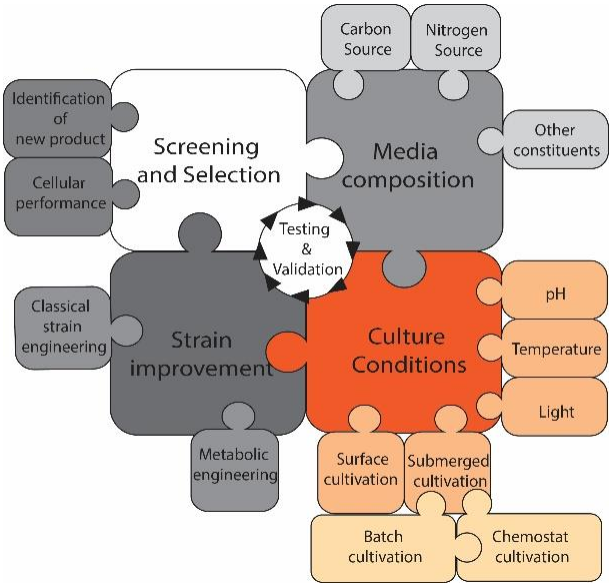


Figure 5.1 Iterative Process of implementing novel cell factories for pigment production; culture condition are highlighted

5.1 Controlling secondary metabolite production by process control

The stringent mechanisms of secondary metabolite biosynthesis in filamentous fungi can limit their production under controlled laboratory conditions as understanding of the detailed regulatory pathway is often incomplete. Even so, since the secondary metabolite profile of filamentous fungi is heavily affected by culture conditions, fermentative production of these metabolites can be increased by adapting the process parameters to the physiological needs of the fungus (H. Y. Kim et al., 2016). Process parameters refer to culture conditions such as pH, temperature and light, as well as cultivation modes like submerged fermentation (SmF) in batch or chemostat mode, or solid-state fermentation (SSF) (see Figure 5.1). Metabolically, the effects of varying pH and temperature are associated with changes in the activities of proteins. By altering those culture parameters, activities such as cellular growth and production of primary and secondary metabolites (Méndez et al., 2011) can be controlled. Most process parameters are usually kept constant throughout the cultivation process (J. Nielsen, 2006).

5.1.1 Submerged vs surface cultivation

As discussed in the introduction in section 2.6.1 "**Type of cultivation**" (p. 20), possible cultivation methods for production of azaphilone pigments are submerged and solid state (surface) cultivation. *Monascus* Pigments (MP) are typically produced by SSF (J C Carvalho et al., 2007; Johns & Stuart, 1991) with the advantage that the products of SSF can directly used as food colorants in Asia (Liu et al., 2010). Products of submerged cultivation need to be extracted from the media before use. General advantages and disadvantages of SmF and SSF are summarized in Table 5.1.

T. atroroseus has been reported to produce red pigment both in submerged and in solid state cultivation (Mapari, 2008). One way of surface- growth cultivation is to use thin sheets of cellophane on top of liquid medium (Harmsen & Kolff, 1947). The cellophane acts like a membrane, because it is impermeable to the fungus, but allows passage of water and nutrients. It represents an interesting cultivation technique for studying secondary metabolite production in solid state cultures with the advantage of easy product recovery.

Depending on the cultivation method, differences in metabolite production, gene expression and protein secretion patterns have also been reported in other fungal cell factories (Biesebeke, 2002; H. Y. Kim et al., 2016). All that indicates the choice of cultivation mode might influence the pigment production profile in *T. atroroseus.*

Table 5.1. Advantages and disadvantages of surface growth vs. submerged growth in liquid culture

	Advantages	**Disadvantages**
Surface growth on liquid culture (SSF)	Easy, minimal equipment required	Growth is limited by surface size and water
	Screening of several media constituents possible	Limited control (heat, pH)
		Low information output- no biomass data or growth rate information
		Poor reproducibility- for inoculation one spore/well
		Gradients in temperature and nutrient concentration
Submerged growth in liquid culture (SmF)	Reproducible- exact spore concentration can be calculated	Time and Labor intensive- more preparation is needed
	High information output, pH and dry weight data available	
	High process control	
	Good mixing	
	Growth is mostly carbon limited and fungus is following batch growth curve	

5.1.2 Chemostat vs. batch cultivation

Batch, fed-batch and chemostat are different operation modes for submerged cultivation in bioreactors and are described in the introduction in 2.6.1.2 "**Submerged cultivation of filamentous fungi in the bioreactor"** (p. 21).

Batch cultivation is the simplest configuration among stirred tank reactors. During this type of cultivation, no nutrient feed is added or removed.

In a chemostat cultivation, fresh medium is continuously added, while culture liquid that contains left over nutrients, metabolites and microorganisms is continuously removed. This happens at the same rate to keep the culture volume constant (Novick & Szilard, 1950). An equilibrium between population growth and loss by overflow can thus be obtained and is called steady state. Growth of the microorganism is controlled, by the presence of one single growth-limiting factor, usually the carbon source. All other nutrient factors are in excess. The specific growth rate of the microorganism can be altered, by changing the feeding rate of the medium into the bioreactor. The ratio between the liquid feed rate (ν, $L \cdot h^{-1}$) and the medium volume, (V, L) is called dilution rate and is shown in Eq. 5.1. The dilution rate should not exceed the maximum growth rate of the microorganism (James, 1961).

Eq. 5.1 $$D = \frac{\nu}{V}$$ (dilution rate)

Continuous fermentation usually involves two stages: start up and steady state. During the start-up period, the fermenter is operated in batch mode until cells grow to the desired cell concentration. Once the cell culture is established, continuous operation starts. In steady state, the volume inside the bioreactor is constant and the medium is perfectly mixed. That means that as long as all input variables (O_2, cultivation medium, pH, temperature, stirring, etc.) are kept constant, all output variables (biomass, CO_2- exhaust, products) remain constant too. Major risks of continuous cultivation are the high possibility of contamination and wash out of cells at too high dilution rates. In addition, the final product concentration is generally lower than in batch or fed-batch fermentations, which increases the cost of product recovery (John Villadsen, Jens Nielsen, 2011).

Continuous cultivation represent a good tool for quantitative physiological analysis and can help to understand the pigment production mechanism in *T. atroroseus*.

5.1.3 Temperature

For most microorganisms, temperature is a critical environmental factor for regulating developmental and physiological processes. The specific growth rate (μ_{max}) of a microorganism tends to increases with temperature up to a certain point where denaturation of enzymes starts. Beyond that point, performance of the cell decreases rapidly. A typical thermal performance curve is illustrated in Figure 5.2.

Monascus spp. are typically cultured at 25–30 °C (Joshi et al., 2003). Nonetheless it has been reported that low temperatures (25 °C) can promote a ten-fold increase in pigment yield compared to yields at higher temperatures (30 °C) (Ahn et al., 2006). This could be explained by slower cell growth and improved homogeneity in the fermenter, better oxygen transfer and lower viscosity. *T. atroroseus, P. purpurogenum* DPUA 1275, *P. purpurogenum* GH2 and other related species are typically cultivated at 25 or 30° C (Babitha et al., 2007a; Gunasekaran & Poorniammal, 2008; Mapari, Meyer, Thrane, et al., 2009; Ogihara & Oishi, 2002; Santos-Ebinuma et al., 2014).

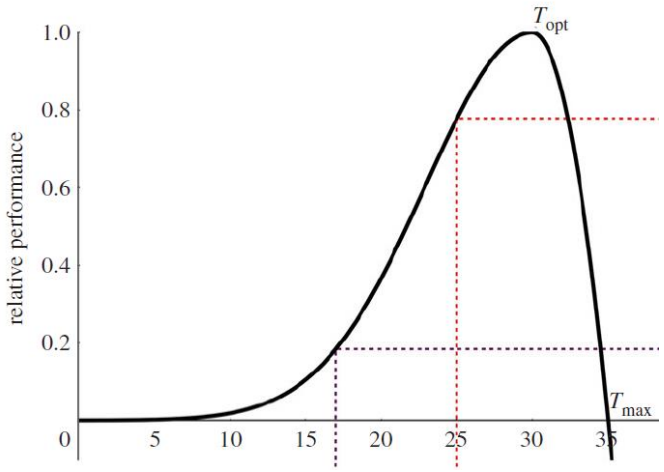


Figure 5.2 Typical thermal performance curve. T_{opt} marks the temperature at which performance is greatest and T_{max} marks the critical transition to negative values at high temperatures (Vasseur et al., 2014)

5.1.4 pH

The influence of pH on the cellular and metabolic activity is determined by the sensitivity of the individual enzymes to changes in pH, because enzymes are

usually only active within a certain pH interval. It has been reported that different pH pattern during submerged cultivation of *Monascus* affected both growth and pigment production (Carels & Shepherd, 1978). Generally, the suitable pH for growth and pigment production of *Monascus* spp. is 5.5–6.5 (Feng et al., 2012). However, changes in pH may influence production of single MP constituents, for example ankaflavin (yellow MP) synthesis by *M. purpureus* was favored at pH 4.0 (M. Chen & Johns, 1993). pH might also affect formation of conidia, sporulation, thereby influencing growth and pigment production (Carels & Shepherd, 1978). By increasing the pH of the cultivation medium, the reaction between orange pigments and amino-group containing compounds forming red-colored complexes is stimulated. Conversely, a low pH prevents the nucleophilic addition of amino groups to the oxygen atoms of orange pigments and consequently red pigment formation was limited while presence of orange MP in the medium was enhanced (Patakova, 2013). A two-stage cultivation conducted at two different pH values (pH 5.5 and pH 8.5) resulted in increased pigment production in *Monascus purpureus* (Orozco & Kilikian, 2008). When grown in submerged conditions, optimal pH for pigment production with *P. purpurogenum GH2* was found to be pH 5 (Méndez et al., 2011). Studies with *P. purpurogenum* DPUA 1275 demonstrated also that a pH of 5 is favorable for pigment production (Santos-Ebinuma et al., 2014).

5.2 Experimental strategy

As this thesis is primarily focusing on process design for submerged cultivation, only very few experiments were carried out propagating *T. atroroseus* in surface cultivation. However, for investigation of the possibility, a small scale experiment in 12- well plates on cellophane was conducted.

Furthermore, it was assessed how temperature and pH affect submerged performance of *T. atroroseus* by testing different conditions in bioreactors. The medium recipe proposed in Chapter 4 was used and only culture parameters were altered. The influence of variations in temperature was investigated in an interval from 20 °C to 35 °C in order to determine a suitable cultivation temperature. Using this temperature optimum, a wide range of pH was screened (from pH 3 to pH 7) to study the effect of pH on the pigment profile of *T. atroroseus*. Chemostat cultivation were carried out at pH 3 and pH 5, assessing the possibility of growth in steady state. The experimental strategy for this chapter is illustrated in Figure 5.3.

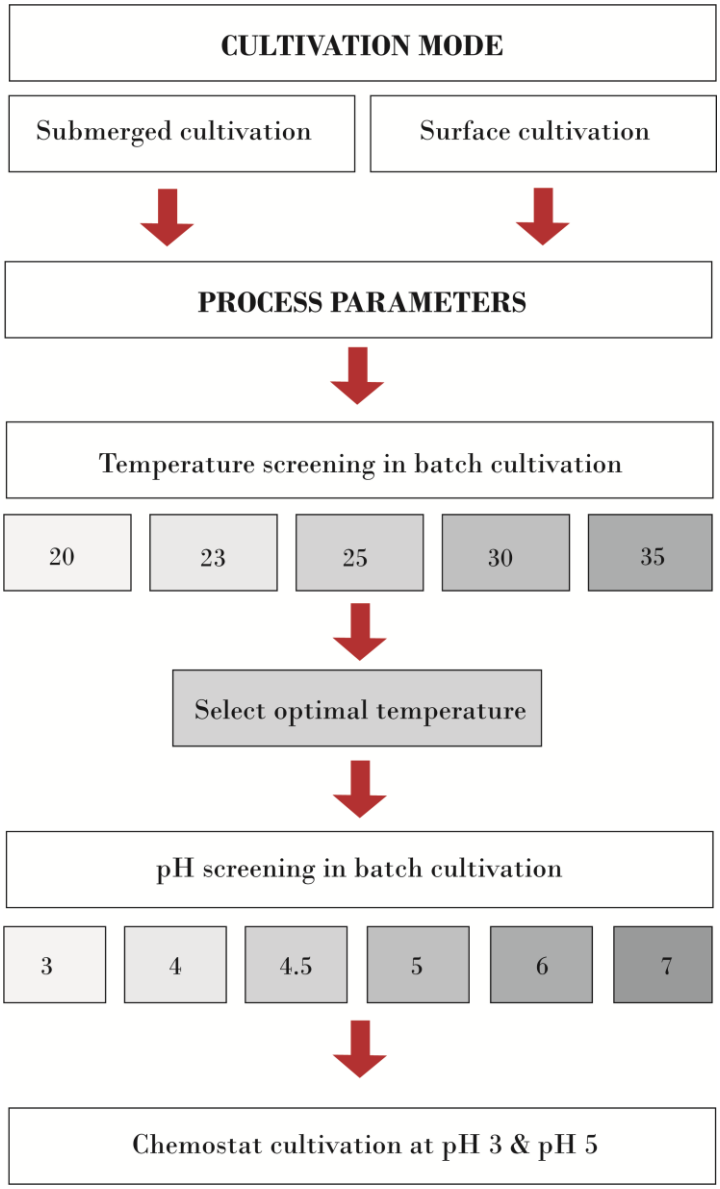


Figure 5.3 Experimental strategy for process design

5.3 Material and Methods

A detailed description of spore preparation, bioreactor process details and sampling can be found in section 3 "**Materials & Methods**" (p. 29).

Surface cultivation (Cellophane studies)
For surface cultivation on cellophane and the control as standard submerged cultivation, potassium nitrate was replaced by 0.1 mol monosodium glutamate as nitrogen source. Both cultivations had an initial pH of 5. For surface cultivation, the fungus was cultivated in a 12- well plate with 5 mL liquid medium per well. A piece of cellophane with a diameter of 1 cm was sterilized with ethanol and then placed on top of the liquid culture medium of each well. The spores for the cellophane cultivation were harvested and counted as described in section 3 "**Materials & Methods**". Each well was then inoculated with max. 10 spores/well and incubated for 168 h at 25 °C.

Trace metal solution: $CuSO_4 \cdot 5\ H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.04 g/L), $FeSO_4 \cdot 7\ H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2\ H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7\ H_2O$ (8 g/L).

Temperature screening: Cultivation were performed in 0.5 L bioreactors with a working volume of 300 ml and the following medium: Sucrose (7.5 g/L), Glucose (1 g/L), KNO_3 (10 g/L), KH_2PO_4 (10 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), NaCl (1 g/L), KCl (0.5 g/L), CaCl (0.1 g/L) and 2 ml/L of trace metal solution.

The cultivation was kept at pH 5 with 1 vvm and 800 rpm. For the temperature screening, growth rate calculations are based on accumulated CO_2 exhaust values. More details about the calculation can be found in section 3.10.1."**Overall CO2-production in batch cultivation**" on p. 33.

pH screening: Experiments were performed in 1L bioreactors with the following media compositions: Sucrose (7.5 g/L), Glucose (1 g/L), KNO_3 (10 g/L), KH_2PO_4 (10 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), NaCl (1 g/L), KCl (0.5 g/L), CaCl (0.1 g/L) and 2 ml/L of trace metal solution.

The cultivation was kept at 30 °C with 1 vvm and 800 rpm. Growth rate calculations are based on dry weight measurements.

Cultivation with pH switch: Cultivation were carried out in Sartorious 1 L with the following medium: Sucrose (20g/L), Glucose (1g/L), KNO_3 (10 g/L),

KH_2PO_4 (10 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), NaCl (1 g/L), KCl (0.5 g/L), CaCl (0.1 g/L) and 2 ml/L of trace metal solution.

The initial pH of the cultivation was pH 3. After 55 h of cultivation, the pH was switched to pH 4.5 The cultivation was performed at 30 °C, 800 rpm, 1 vvm.

Chemostat cultivation: Chemostats were performed in 1L bioreactors with the following medium: Sucrose (7.5 g/L), Glucose (0.375 g/L), KNO_3 (10 g/L), KH_2PO_4 (10 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), NaCl (1 g/L), KCl (0.5 g/L), CaCl (0.1 g/L) and 2 ml/L of trace metal solution.

The dilution rate of the chemostat was D= 0.07 h^{-1}. The cultivation was performed at 30 °C, 800 rpm, 1 vvm.

Pigment yield was determined by measuring the absorbance at 500 nm and converting the value into g/L with the help of the standard curve from atrorosin-E shown in Figure 3.2 on p. 37.

5.4 Results and Discussion

5.4.1 Surface growth

In Chapter 6 " **Tailored pigment production in *T. atroroseus***" on p. 97, a cultivation method for production of pure azaphilone pigments in *T. atroroseus* is described. The proposed medium recipe from chapter 6 was used to determine if there was a difference in the pigment production portfolio of *T. atroroseus* between solid state and submerged cultivation. *T. atroroseus* was propagated as surface culture and submerged culture with monosodium glutamate (MSG) as sole nitrogen source. For both cultivations a liquid medium was used, but for the surface cultivation a piece of cellophane on top of the liquid served as solid substrate. The piece of cellophane assured radial, surface growth of the fungus while enabling excretion of the pigments into liquid media and thus simplifying extraction of the pigments.

In both cultivation types, *T. atroroseus* excreted red pigments into the cultivation medium. However, there were differences in morphology. When cultivated in surface culture the colonies of *T. atroroseus* had the typical greenish color of filamentous fungi caused by the fungal spores (see Figure 4.4 A)), but when cultivated submerged, the mycelium was colored intensively red (see Figure 4.4 B).

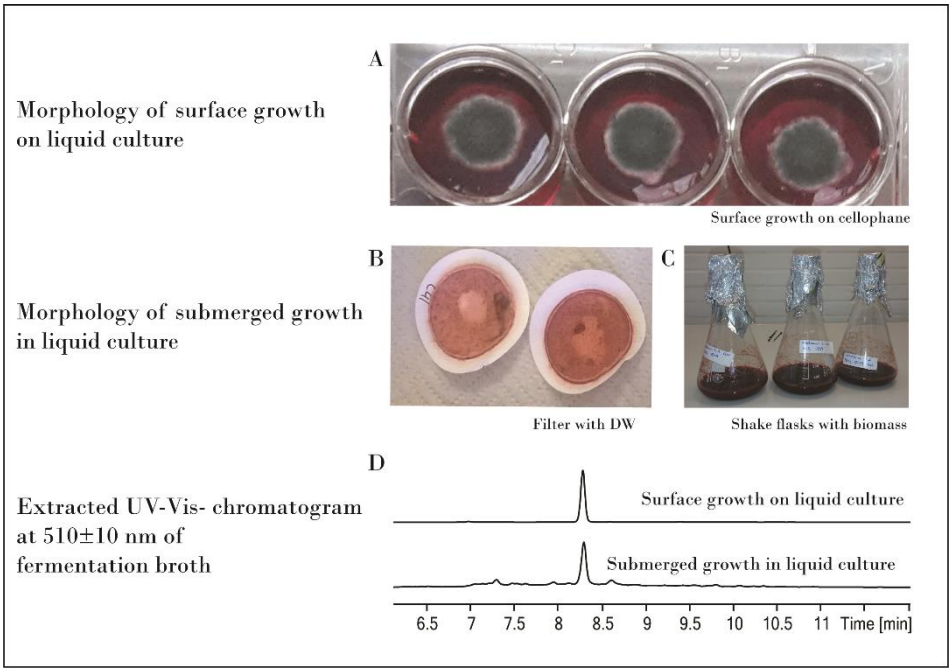


Figure 5.4 Differences in fungal morphology in submerged and surface culture A) Surface growth in liquid culture with MSG as nitrogen source B) Filter with DW from submerged growth with MSG as nitrogen source C) Shake flasks with MSG as nitrogen source D) UV-Vis-chromatograms at 520±10 nm showing the presence of the same red pigment in both cultivation

In both, SmF and SSF, red pigments are excreted into the media (see Figure 4.4 C). UV- Vis chromatography showed a similar color profile for both cultivation techniques, indicating that the same major pigment was produced (Figure 4.4 D). This means that the cultivation mode does not appear to influence the pigment production profile of the fungus. Quantitative assessment of the produced pigment was not possible, because in the cellophane culture, the fungus overgrew the well, before carbon limitation of the medium was reached. Therefore, direct comparison of pigment yield was not possible. Also, determination of process parameters such as growth rates and yields could not be achieved in surface cultures. Cellophane cultivation in 12-well plates could represent an excellent tool for early screening experiments, but for the investigation of process parameters in this chapter, submerged growth of *T. atroroseus* was favored over surface growth. Submerged cultivation assure better process control and higher information level.

5.4.2 Temperature screening

For the temperature screening, cellular performance of *T. atroroseus* was investigated in the bioreactor at 20, 23, 25, 30 and 35 °C. Physiological parameters like the specific growth rate μ_{max} and cultivation time were determined and the pigment production yield Y_{SP} in c-mole/c-mole was calculated for each of the cultivation. The growth rate μ_{max} was determined based on accumulated CO_2-exhaust, because due to the working volume only being 300 mL, accurate determination of DW-based μ_{max} could not be achieved.

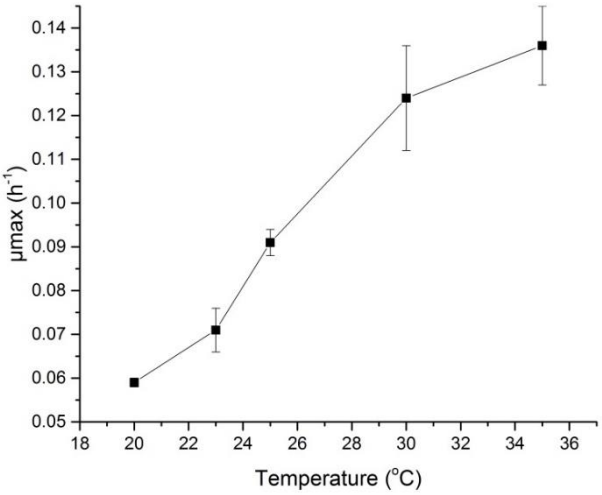


Figure 5.5 Growth rates μmax of *T. atroroseus* in submerged cultivation at different temperatures

Figure 5.5 shows that the growth rate, based on accumulated CO_2-exhaust , of *T. atroroseus* increased with temperature. By increasing the temperature from 20 to 35 °C, the growth rate more than double (from 0.06 h^{-1} to 0.136 h^{-1}). It was also observed, that an increase in temperature led to, not only a shortened lag-phase, but to an overall faster process. The length of the individual cultivation at different temperatures can be found in Table 5.2. An increased cellular growth at higher temperatures is a common phenomenon in filamentous fungi (Reichl et al., 1992; Trinci, 1969).

Table 5.2 Cultivation length (h) of submerged cultivation of *T. atroroseus* at different temperatures. Experiments were performed in 0.5 L bioreactors, at pH 5 with 800 rpm and 1 vvm

Cultivation temperature (°C)	20	23	25	30	35
Cultivation length (h)	170	140	130	100	90

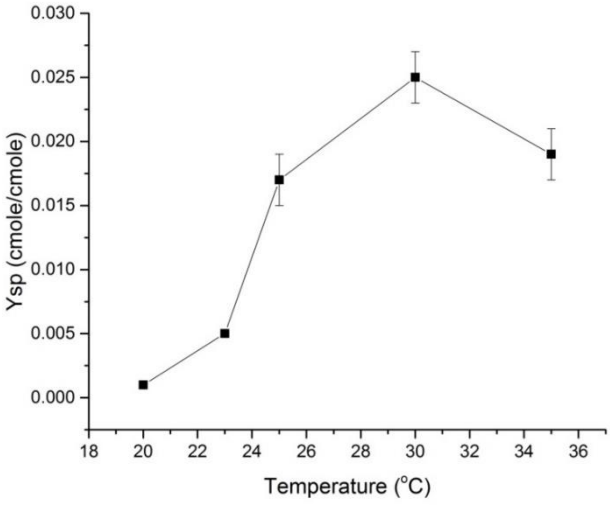


Figure 5.6 Y_{SP} in c-mole of pigment/c-mole of biomass of *T. atroroseus* in submerged cultivation at different temperatures. Bioreactor experiments were performed in 0.5 L reactors, at pH 5 with 1 vvm and 800 rpm.

The yield coefficient Y_{SP} in relation to the cultivation temperature is shown in Figure 5.6. Pigment production yields followed the trend of the growth rate, but then peaked with a yield coefficient Y_{SP} of 0.025 c-mole/c-mole at 30 °C and decreased thereafter. Changes in temperature are known to affect metabolite production by enzymes (Gmoser et al., 2017), The drop in pigment yield at 35 °C might indicate that enzymes involved in azaphilone biosynthesis in *T. atroroseus* have reduced activity at higher temperatures than 30 °C. For *Monascus*, suitable temperatures for pigment production vary between 25 °C (SmF) (Ahn et al., 2006), 30 °C (SSF) (Babitha et al., 2007a), 32 °C (SmF) (Zhang et al., 2013) and 35 °C (SSF) (Zhang et al., 2013). These results suggests differences in performance depending on *Monascus* species and cultivation mode. In *P. purpurogenum*, red pigment production was found to be best at 30 °C (SmF) (Santos-Ebinuma et al.,

2014). Since the purpose of this study was to find a suitable temperature for pigment production, all further experiments were conducted at 30 °C.

5.4.3 pH study

Many filamentous fungi in submerged cultivation respond to changes in pH by an altered cellular performance in terms of growth or metabolite production. In Chapter 4.4.3 "**Screening of nitrogen source on sucrose**" on p. 49 growth of *T. atroroseus* without pH control was described. When using $(NH_4)_2SO_4$ as nitrogen source in shake flask cultivation, a drop in pH from 5 to 2.5 and yellow/orange pigment production was observed. When cultivated at controlled pH 5 in the bioreactor however, *T. atroroseus* produced red pigments on $(NH_4)_2SO_4$. These findings suggested an important impact of pH. Here, several values of pH (3, 4, 4.5, 5, 6, and 7) were tested for their influence on pigment production and growth.

5.4.3.1 pH study on KNO_3

Studying cellular performance of *T. atroroseus* at different pH, revealed converse results with regards to growth and pigment production. Figure 5.7 shows growth rates of *T. atroroseus* in the range from pH 3 to pH 7. Here, growth rates are based on DW measurement.

The highest growth rate of *T. atroroseus* was measured at pH 3 (0.102 ± 0.008 h^{-1}). Thereafter μ_{max} slowly decreased with an increase in pH. At pH 7, the growth rate was 10 fold lower with 0.012 h^{-1}. Physiological growth conditions were thus best for *T. atroroseus* at pH 3.

When looking at the conversion of substrate into biomass and pigments, it wasn't possible to come to such a clear conclusion. The yield coefficients Y_{SX} and Y_{SP} are shown in Figure 5.8 At pH 3, the cellular growth performance was best. A yield coefficient Y_{SX} of 0.581 ± 0.011 c-mole/c-mole was calculated, indicating that almost 60% of the carbon source sucrose was converted into biomass. This is in accordance with the high growth rate determined at pH 3. However, no pigment production could be detected. At pH values between 4 and 5, Y_{SX} dropped to around 0.4 c-mole/c-mole, but pigment production was high with a maximum Y_{SP} of 0.015 ± 0.001 c-mole /c-mole at pH 4.5 (Figure 5.8). At pH values above 6, both growth and pigment production was low indicating that above pH 6, cellular performance of *T. atroroseus* was hampered.

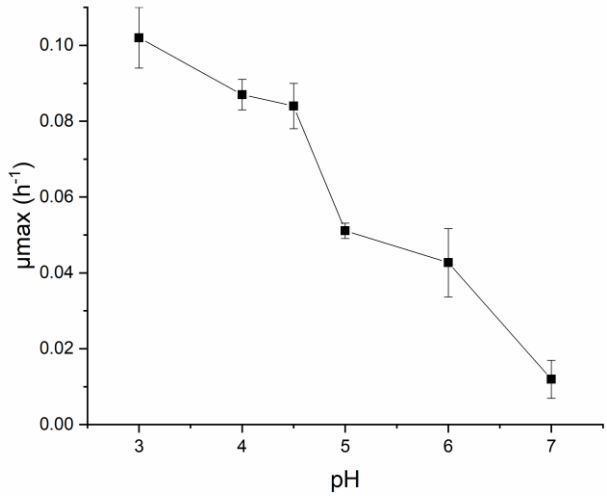


Figure 5.7 Growth rates μ_{max} of *T. atroroseus* in submerged cultivation at different pH values. Bioreactor experiments were performed in 1 L reactors, at 30 °C, with 1 vvm and 800 rpm.

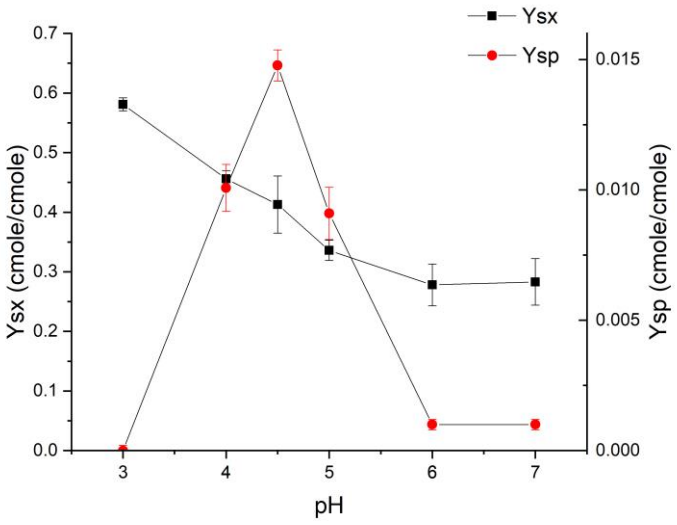


Figure 5.8 Yield coefficients Y_{SX} and Y_{SP} from submerged cultivation of *T. atroroseus* with KNO_3 at pH ranging from 3 to 7. Bioreactor experiments were performed in 1 L reactors, at 30 °C, with 1 vvm and 800 rpm.

Surprisingly, pigment production of *T. atroroseus* peaks at 4.5, while Monascus is cultivated at pH 5.5.-6.5 (Feng et al., 2012). It seemed that *T. atroroseus* prefers a more acidic environment than *Monascus*.

These results suggested a metabolic trade-off between pigment production and growth. Clearly, at low pH, most of the carbon was used for growth and *T. atroroseus* propagated well and fast. At pH 4, the amount of carbon, that goes into forming biomass was reduced. *T. atroroseus* appeared to experiences some kind of stress that induces pigment production as a stress response. Growth was slower than on pH 3, and less biomass was produced. Growth decreased further at pH 4.5, pigment production reached its maximum and thereafter decreased again. At pH 5, both, pigment production and cellular growth is thus reduced compared to pH 4.5.

It can be concluded that pH values favorable for growth do not support pigment production. pH 3 seemed best for growth of *T. atroroseus* , but pigment productions was highest at pH 4.5

The hypothesis of *T. atroroseus* experiencing metabolic stress at pH values higher then 3, could be further strengthened by comparing the CO_2-production pattern from cultivation at different pH values (Figure 5.9). CO_2 production was measured via mass spectrometry throughout the entire cultivation. An increase in exhaust CO_2 can be correlated to growth and therefor the CO_2 production pattern often follows the growth curve of the microorganism in question. At pH 3, the CO_2-exhaust followed a classical exponential batch curve and *T. atroroseus* produced 17.1±0.4 g/L CO_2. Overall CO_2- production was lower at pH 4 and pH 5 (16.5±0.9 g/L and 11.2±0.5 g/L respectively) and did not follow a classic exponential course. For pH 4 and pH 5, the experiments showed initial exponentially CO_2-exhaust, which then after 60 h rather stayed steady over a longer period of time. This observed plateau correlated with pigment excretion and could indicate metabolic differences of *T. atroroseus* in carbon utilization und CO_2-production at pH3 compared to pH5.

Whether pH is the factor inducing the metabolic shift, and thereby triggering pigment production in *T. atroroseus*, remains speculative at this time point.

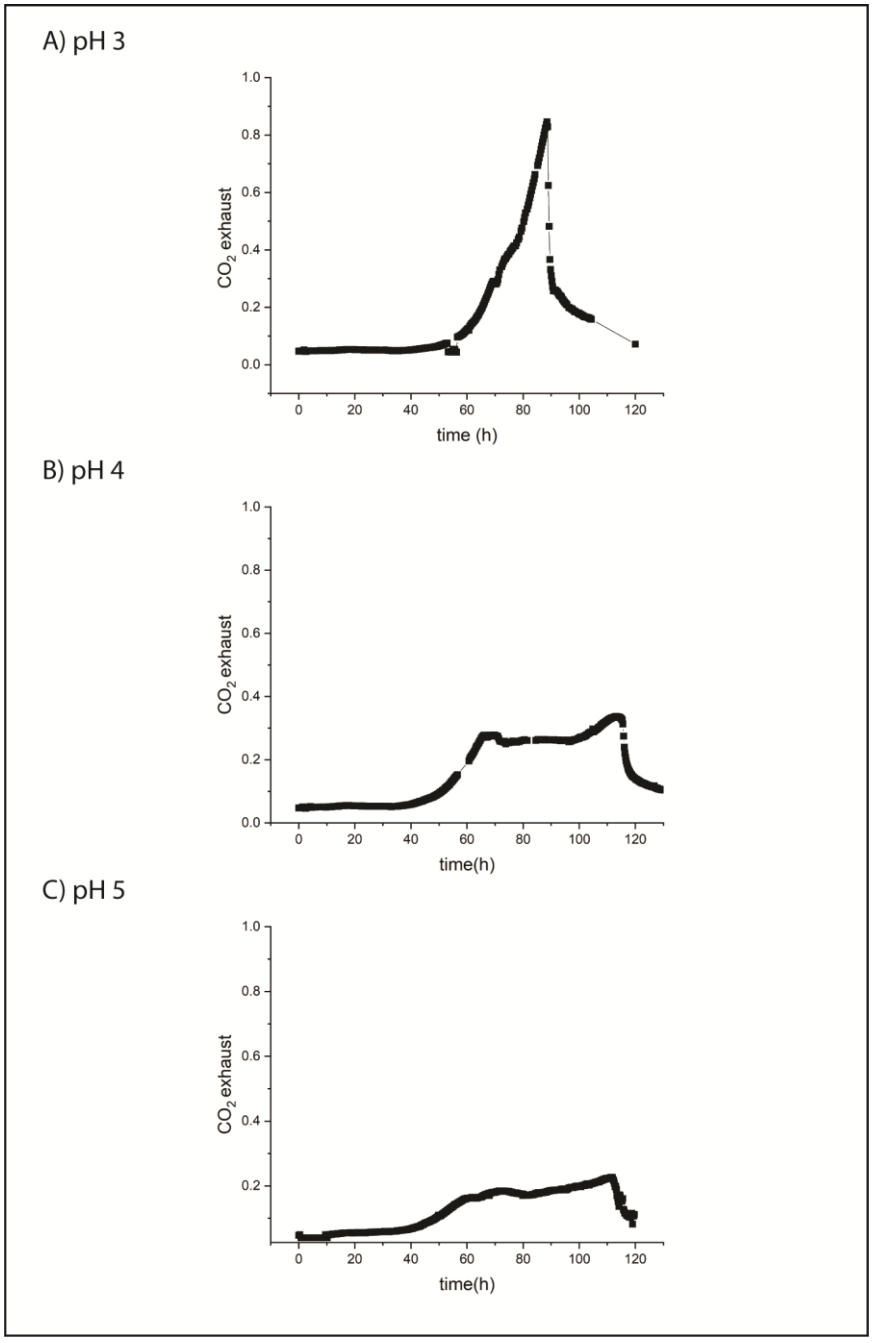


Figure 5.9 CO_2 exhaust pattern from cultivation on KNO_3 at pH3, pH4 and pH5

5.4.3.2 pH study on $(NH_4)_2SO_4$

pH screening experiments at pH 3, 4, and 5 were also performed with $(NH_4)_2SO_4$ as nitrogen source and resulted in similar conclusions as with KNO_3. The results are summarized in Table 5.3. The highest growth rate was seen at pH 3, but as with KNO_3, no pigment production could be detected at this pH. Pigment production was seen at pH 4 and 5, but was overall lower than with KNO_3.

Table 5.3 Yield coefficients Y_{SX}, Y_{SP} and Y_{XP} from submerged cultivation of *T. atroroseus* with $(NH_4)_2SO_4$ at pH 3, 4 and 5. Bioreactor experiments were performed in 1 L reactors, at 30 °C, with 1 vvm and 800 rpm.

	Y_{SX} c-mole /c-mole	Y_{SP} c-mole /c-mole	Y_{XP} c-mole /c-mole	$\mu_{max\ DW}$ (h^{-1}) c-mole /c-mole
pH3	0.524±0.01	No pigment production		0.116±0.011
pH4	0.513±0.02	0.005±0.001	0.010±0.003	0.056±0.002
pH5	0.371±0.109	0.006±0.002	0.016±0.001	0.053±0.006

Also with $(NH_4)_2SO_4$ as nitrogen source, a clear trade-off between pigment biosynthesis and biomass accumulation was observed. No pH supported both, growth and pigment synthesis of *T. atroroseus* equally well.

5.4.3.3 KNO_3 vs. $(NH_4)_2SO_4$

When comparing KNO_3 and $(NH_4)_2SO_4$, *T. atroroseus* performed similar at pH 3 in terms of growth and growth rates of ~0.1 h^{-1} were reached on both nitrogen sources. Growth rates for pigment producing *Monascus* species are generally found to vary from 0.02 h^{-1} for *Monascus* spp. (Krairak et al., 2000) to 0.04 h^{-1} for *Monascus ruber* (M. Chen & Johns, 1993; Hassan Hajjaj et al., 1997; Said et al., 2014). For *Talaromyces*, at the current time only growth rates for cultivation in micro titer plates are available and haven been reported to be 0.067 h^{-1} (Knudsen, 2015). In comparison with other industrial relevant cell factories, namely *Saccharomyces cerevisiae* (μ_{max} up to 0.4 h^{-1} at pH 5 (Van Hoek et al., 1998)) and *Aspergillus niger* (μ_{max} of 0.21 h^{-1} at pH 4.5 (Andersen et al., 2009)), *T. atroroseus* grows relatively slowly. Throughout all tested pH values, biomass accumulation was slightly

improved on $(NH_4)_2SO_4$ and higher Y_{SX} values were reached. This is probably due to the nitrate assimilation pathway described in Chapter 4.

On KNO_3, pigment production was good between pH 4 and pH 5 on KNO_3 and Y_{SP} values around 0.01 c-mole/c-mole were reached. The maximal value was detected at pH 4.5. On $(NH_4)_2SO_4$, only pH 5 supported high pigment production with a yield coefficient Y_{SP} around 0.006 c-mole/c-mole.

With regard to an efficient pigment production process, it should be favorable to have high biomass concentrations. It is assumed that a high cell concentration promotes increased product formation. In the case of *T. atroroseus*, high biomass and enhanced product formation cannot be optimally obtained at the same pH

Therefor the next step was to investigate, if it would be possible to split the process into two phases: into phase 1 at pH 3 to favor propagation of the fungus and into phase 2 at pH 4.5 to induce pigment production. For this experiment, KNO_3 was selected as nitrogen source because it seemed to promote pigment production better than $(NH_4)_2SO_4$.

5.4.4 Induction of pigment production by pH switch

After establishing the important effect of pH on growth and pigment production in *T. atroroseus*, it was investigated, if a pigment production process could be designed, where both, first growth and then pigment production was promoted. This set-up contained a pH-switch from 3 to 4.5. In order for this set-up to be successful, it was important to switch pH before glucose depletion, to ensure that adequate substrate for synthesis of pigment would be available. In order to prolonged the exponential growth phase, 20 g/L of sucrose were used instead of 7.5 g/L. Cultivation profiles for batch cultivation with *T. atroroseus* at pH 3 (A), pH 4.5 (B) and with a pH switch from pH 3 to pH 4.5 (C) are shown in Figure 5.10.

At pH 3 (Figure 5.10 A), the batch cultivation took around 70h and the CO_2 exhaust pattern followed a classical batch curve as described in section 5.4.3.1 "**pH study on KNO_3**" on p. 72. However, no pigment was produced throughout the entire cultivation. Both CO_2 production and biomass accumulation started after around 40 h and carbon depletion was already reached 15 h later. CO_2 production and biomass accumulation peaked shortly afterwards.

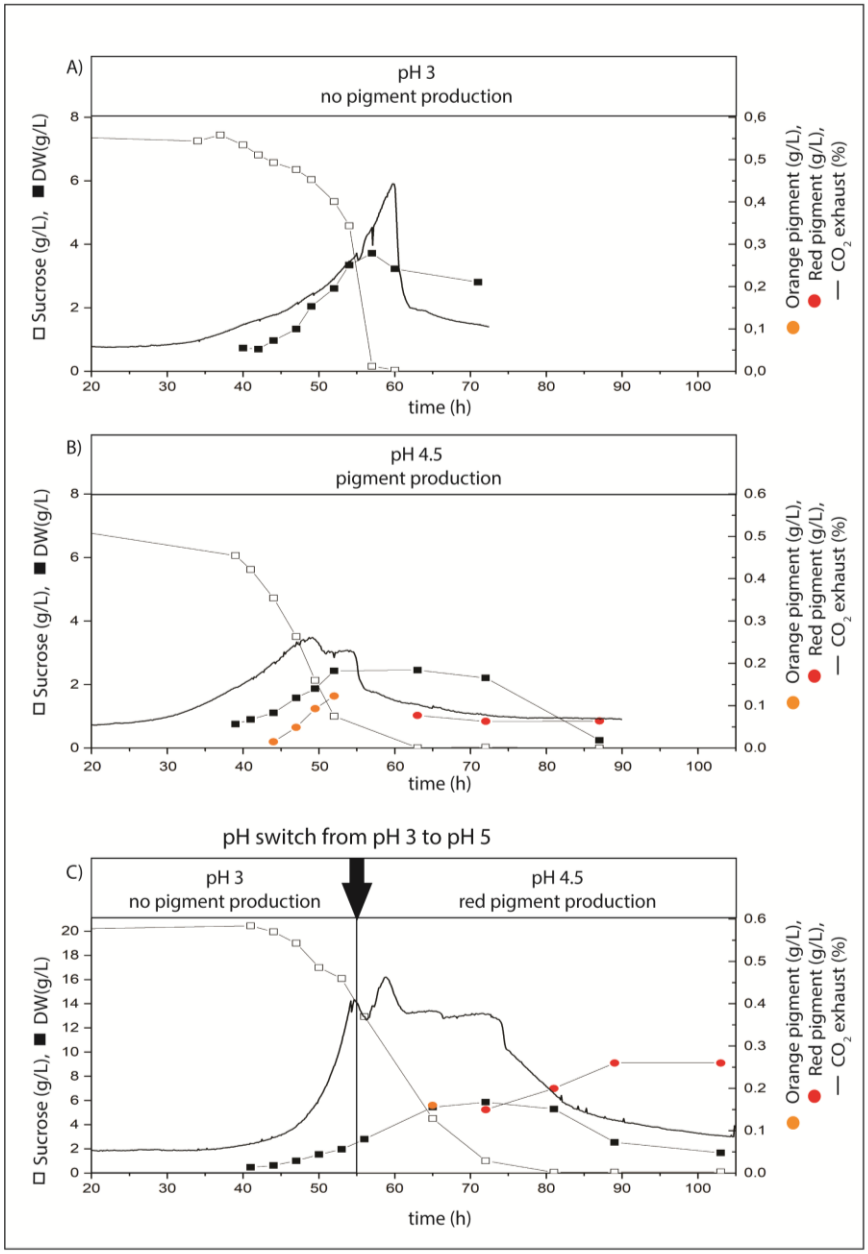


Figure 5.10 Cultivation profile for batch cultivation with *T. atroroseus* at pH 3, pH 4.5 and in the pH-switch cultivation. Bioreactor experiments were performed in 1 L reactor at 30 °C, with 1 vvm and 800 rpm.

At pH 4.5 (Figure 5.10 B), CO_2 production and biomass accumulation started also after a lag phase of 40h. After 45h, production of PP-O started and continued until depletion of carbon source. After the depletion of sucrose and decrease in CO_2 production, red pigment production commenced. LC-MS analysis showed that the orange pigment PP-O was converted into red pigments after the exponential growth phase. A mixture of different red unknown pigments could be identified in the fermentation broth.

The cultivation comprising a pH switch (Figure 5.10 C), started at pH 3 and followed a similar pattern as cultivation A (Figure 5.10 A). No pigment production was detected as long as the cultivation was kept at pH 3. After 55 h pH was increased to a value of 4.5 by addition of NaOH. CO_2 production did drop after the pH switch and then continued until 60 h, where exponential CO_2 production stalled. CO_2 exhaust seemed to stabilize. Simultaneously, at around 65 h, PP-O production started, and the orange pigments were shortly after converted into a mixture of red pigments. Sucrose was depleted at between 75 and 80 h and CO_2 production also dropped around that time. Production of red pigments kept increasing until 90 h. This indicated, as already observed and described earlier, that pigment production/excretion continued even after depletion of the carbon source.

These experiments showed that pigment production in *T. atroroseus* is strongly pH dependent and that throughout one batch cultivation pigment production could be induced by changing the pH from 3 (no pigment production) to 4.5 (pigment production). Evaluating the process productivity, it was found that cultivation B had a Y_{SP} of 0.014±0.001 c-mole/c-mole whereas cultivation C (employing the pH switch) had a Y_{SP} of 0.019±0.001 c-mole/c-mole. Hence pigment yield could be improved by 35% by first promoting propagation of the fungus and then inducing pigment production. Critical steps in this cultivation process were finding the optimal time point for pH switch to ensure enough time for biomass accumulation before the switch so that enough substrate for pigment biosynthesis was available after the switch.

5.4.5 Chemostat cultivation

In batch cultivation, the exponential growth phase of *T. atroroseus* is rather short and it was observed that pigment production started after exponential growth ceased. Chemostat cultivation offer the study of an organism during exponential growth and were therefore performed to gain further insight in the pigment regulation of *T. atroroseus*. Chemostat cultivation were performed with KNO_3 and $(NH_4)_2SO_4$ as nitrogen source.

5.4.5.1 Chemostat with KNO_3 at pH 3

Chemostat cultivation of *T. atroroseus* at a dilution rate of 0.07 h^{-1} were successfully performed at pH 3. Feed was initiated after 40 h. It can be seen in Figure 5.11, that steady state conditions were achieved after 70 h and could be maintained for 7 residence times. The culture was run under carbon limited conditions. Pigment production was however absent under steady state conditions at pH 3. This is in accordance with batch cultivation at pH 3 previously described.

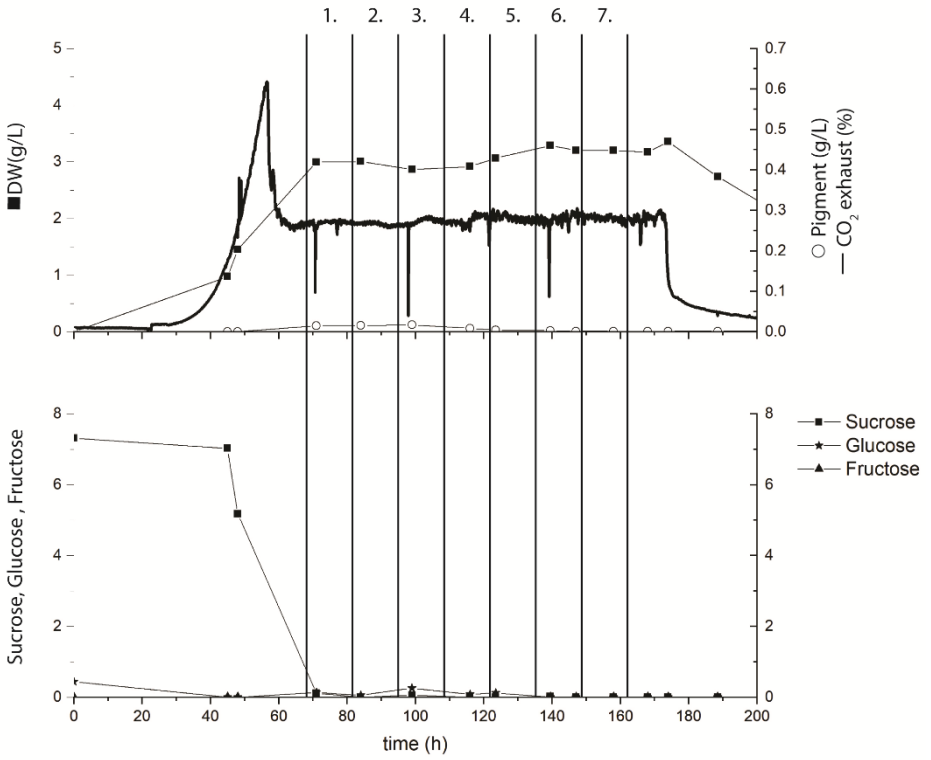


Figure 5.11 Representative chemostat profile for *T. atroroseus* on KNO_3 at pH 3 and a dilution rate of D = 0.07 h^{-1}. Cultivation conditions were 30 °C with 1 vvm and 800 rpm.

5.4.5.2 Chemostat with KNO_3 at pH 5

Chemostat cultivation at a dilution rate of 0.07 h^{-1} were also performed at pH 5. As evident from Figure 5.12, the cultivation did neither reach steady state nor did the cultivation became carbon limited. Residual concentrations of glucose and fructose up to 2 g/L could be detected in the fermentation medium. Feed was initiated after 40 h, at the end of the exponential growth phase. CO_2- production was in general lower then on pH 3. It appeared to stabilize after 3 residence times, but then *T. atroroseus* commenced growth again in residence time 6, resulting in an increase in biomass and CO_2- exhaust. Very low amounts of orange pigments were detected during the 2nd residence time, but LC-MS analysis was inconclusive, due to antifoam disturbing the analysis and no known pigments could be identified. We speculate the orange pigments PP-O being.

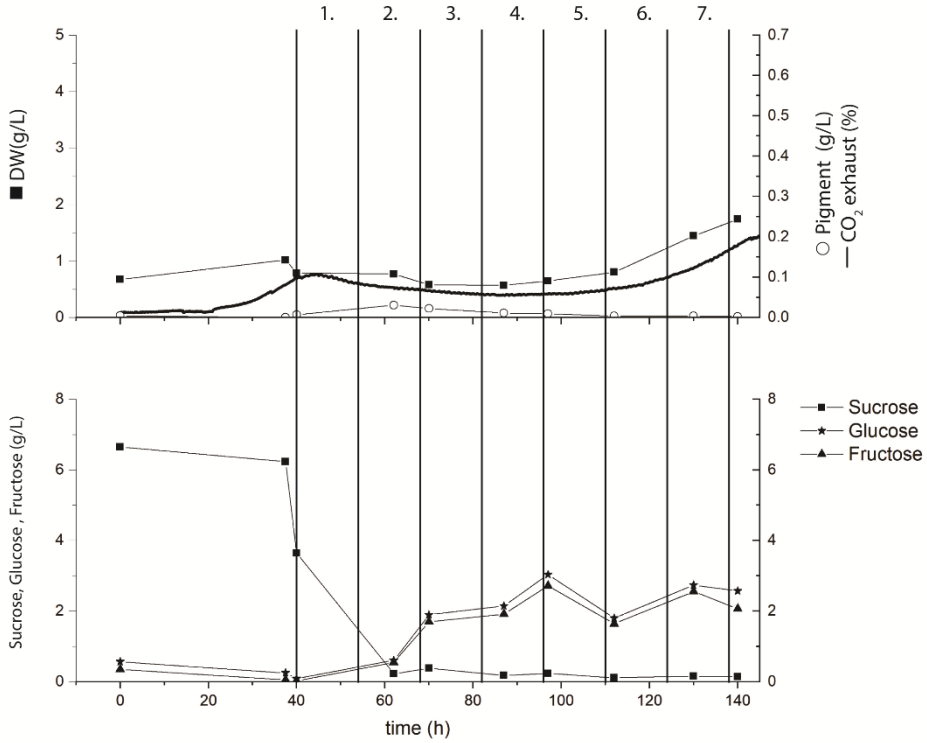


Figure 5.12 Representative chemostat profile for *T. atroroseus* on KNO_3 at pH 5 and a dilution rate of D = 0.07 h^{-1}. Cultivation conditions were 30 °C with 1 vvm and 800 rpm.

The experiment at pH 5 was repeated, with the alteration that, after 130 h (ca. 6 residence times) cultivation mode was switched from continuous into batch mode. This cultivation profile is shown in Figure 5.13. The same tendencies were seen like in the previous experiment. In continuous mode, no steady-state was reached, the cultivation did not become carbon limited and again almost no pigments were produced. After switching into batch mode after 130 h, both CO_2-exhaust and biomass accumulation continued to increase. Carbon was depleted after 140 h of cultivation. Also, production of pigment, first orange and shortly afterwards red, started ca. 10 h after the feed was turned off. The final concentration of red pigments was measured to be 0.1 g/L. These results corroborated, that *T. atroroseus* does not produce azaphilone pigments while being in the exponential phase growth phase.

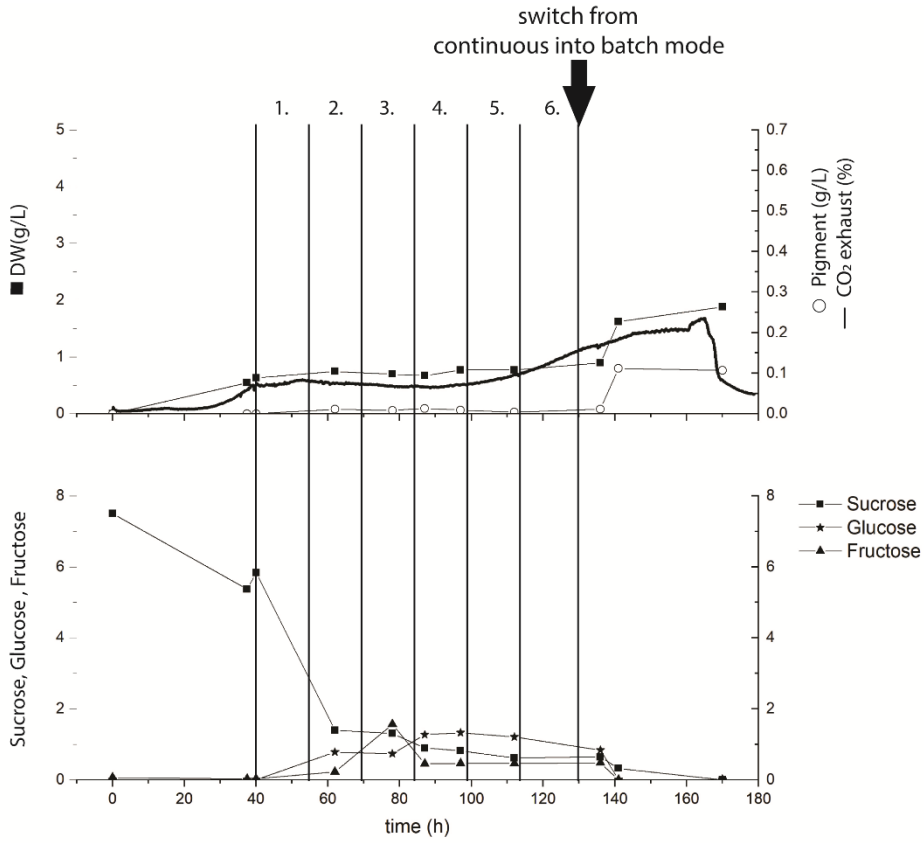


Figure 5.13 Representative chemostat profile for *T. atroroseus* at pH 5 and a dilution rate of D = 0.07 h^{-1}. Switch to batch mode after 130h. Cultivation conditions were 30 °C with 1 vvm and 800 rpm.

It can be concluded that continuous cultivation is not the method of choice for red pigment production in *T. atroroseus*. However, results emphasized the differences in cellular performance at pH 3 and pH 5. At pH 3, *T. atroroseus* performed well and could be maintained in steady state over several residence times. At pH 5, neither steady state nor carbon limitation was achieved with *T. atroroseus*. Lower values of biomass and CO_2- exhaust at pH 5 than at pH 3 indicated stress of *T. atroroseus* at pH 5. That no carbon limitation was reached indicates that the cultivation was limited by something else. Since continuous cultivation were performed with 7.5 g/L of sucrose and 10 g/L of KNO_3, a limitation in nitrogen source during steady state seems unlikely. What the limiting factor was, remains unknown for now.

Furthermore, no red pigment production was observed on neither pH 3 nor at pH 5 in continuous mode. Very small amounts of orange pigments were detected in the chemostat at pH 5. It was only, after the shift from continuous to batch mode, that red pigment production commenced. This can have two possible explanations.

1. *T. atroroseus* must not be in exponential growth in order to synthesize red pigments. Batch cultivation presented in Section 4.4.6 "**Batch cultivation on KNO_3, $(NH_4)_2SO_4$ and NH_4NO_3 at pH 5**" on p. 53 show that red pigment production first started, after exponential growth faded. Pigment production seemed to be dependent on the growth stage of *T. atroroseus* . This has also been previously speculated in the literature to be the case in *Monascus* (Kojima et al., 2016).
2. Nitrogen limitation could be a prerequisite for pigment production in *T. atroroseus*. Nitrogen limiting conditions were only reached after the continuous mode was switched into batch mode. Creating nitrogen limitation might have induced a metabolic shift towards pigment production.

Production of red pigments is known to occur via amination of the orange precursor PP-O. We speculate that production of PP-O is a nitrogen-scavenging mechanism, where PP-O is produced and excreted into the media when nitrogen limitation in the cultivation medium is reached. It then reacts with amino-group containing compounds found in the broth to form red pigments. Since PP-O production is initiated by *T. atroroseus* after the nitrogen concentration has reached a specific threshold, red pigments are supposed to "store" the nitrogen, so that it remains available for the fungus and can be used as nitrogen source, once all

nitrogen is depleted. Red pigment concentration has been seen to decrease again after depletion of substrate (Rasmussen, 2015).

5.5 Conclusion

Red pigment production in *T. atroroseus* occurred both, in submerged and surface cultivation and the same pigments were produced, if media constituents were kept similar in both cultivation methods. Since process control and scalability was limited for surface cultivation, submerged cultivation of *T. atroroseus* was the method of choice for the rest of this thesis.

The investigation of process parameters concluded that the best combination of the tested parameter for red pigment production in *T. atroroseus* was a fixed temperature of 30 °C at a pH of 4.5. Furthermore, a clear trade-off between growth performance and pigment biosynthesis was observed. Cellular growth was best at pH 3, but pigment yields were highest at pH 4.5. These findings were further confirmed by chemostat cultivation at pH 3 and pH 5, whereas *T. atroroseus* suffered some stress at pH 5 and could not be maintained in steady state. No red pigments were produced in continuous culture neither at pH 3 nor at pH 5.

Finally, it was demonstrated, that pH control could be used to induce pigment production in *T. atroroseus* proving the importance of pH control in process design for *T. atroroseus*. By changing the pH during a cultivation, it was possible to promote growth and propagation of the fungus in a first step at pH 3 and then in a second step inducing pigment synthesis by shifting pH to 4.5. By doing so, the yield coefficient (Y_{SP}) could be increased by 35 %- from 0.014 c-mole/c-mole at pH 4.5 to 0.019 c-mole/c-mole.

6 Towards identification of a novel class of azaphilones in *T. atroroseus* in submerged cultivation‡

This chapter describes spectroscopy-guided analysis of the pigment production profile of *T atroroseus*. A novel class of azaphilone pigments called atrorosins was discovered and structurally described. All chemical analyses presented in this chapter were performed by Thomas Isbrandt.

‡ Parts have been adapted from Isbrandt *et al. to be submitted*. "Atrorosins: a new subgroup of *Monascus* pigments from *Talaromyces atroroseus"* and can be found in Appendix C

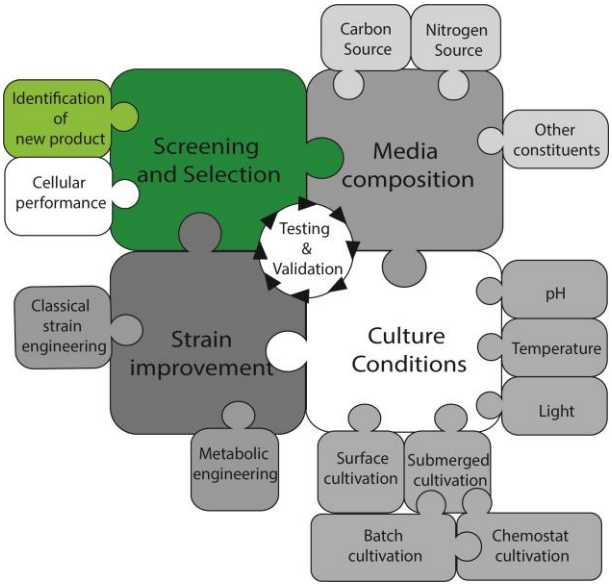


Figure 6.1 Iterative Process of implementing novel cell factories for pigment production; screening for POI is highlighted

6.1 Identification of novel products in fungal cell factories.

In the previous chapters, a reproducible, high yielding pigment production process has been designed, and *T. atroroseus* was physiological characterized. Here, the focus will be on the identification of the red pigments produced by *T. atroroseus* (see Figure 6.1 "Identification of POI"). Detailed chemical analysis of fungal extracts generally aims at the discovery of novel metabolites. One strategy for distinguishing between known and unknown metabolites is called dereplication. Dereplication refers to the combined use of various analytical techniques to identify known compounds based on their chemical properties, such as accurate mass, fragmentation pattern, absorption or emission spectrum, or retention time (Hubert et al., 2017; K. F. Nielsen et al., 2011). The most commonly used techniques, are liquid chromatography (LC), UV-Vis spectroscopy, mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR).

The major steps of novel product identification are highlighted in Figure 6.2. In order to study fungal metabolites and to identify novel ones, they need to be extracted from the fermentation broth (Step 1) and then purified (Step 2). Once the pigments are purified, their structure can be elucidated via NMR (Step 3).

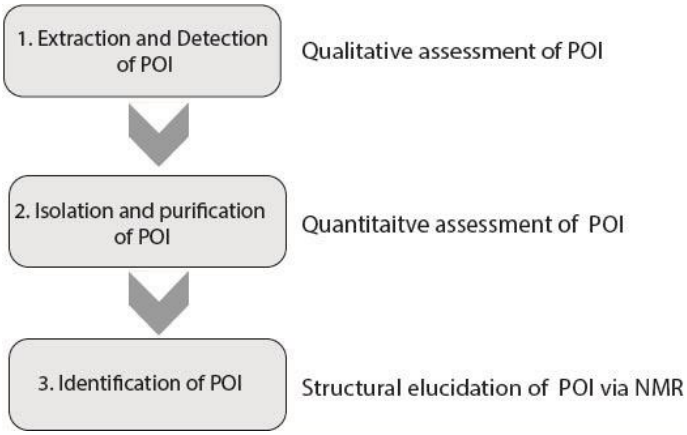


Figure 6.2 Summary of steps for novel product discovery

6.1.1 Qualitative assessment: extraction and detection

6.1.1.1 Extraction

Different extraction methods for MP have been reported, depending on their solubility, as the red MP are more water-soluble than the yellow and orange ones. In order to assess the intracellular pigments, the cells need to be separated from the liquid media before extraction (Broder & Koehler, 1980; Velmurugan et al., 2010). MP can be extracted with methanol (Babitha et al., 2007b) or ethyl acetate (Akihisa et al., 2005; Blanc, Loret, et al., 1995; Julio Cesar de Carvalho et al., 2005; Jang et al., 2014; Wong & Koehler, 1983). MP from solid state fermentation and MP still bound to the mycelium can be extracted through solid-liquid extraction (Hu et al., 2012; Kongruang, 2011; Mohamed et al., 2012) or microextraction (Mapari et al., 2008; Mapari, Meyer, & Thrane, 2009). MP dissolved in the supernatant from a submerged cultivation can be extracted through liquid-liquid extraction (Hu et al., 2012; Velmurugan et al., 2010; Zhou et al., 2009). It was recently published, that the pH control during sample handling should be below pH 2.5 to prevent the creation of artifacts resulting from the reaction of orange pigments with amino compounds present in the fermentation medium (Shi et al., 2016).

6.1.1.2 Detection

Ultraviolet-visible (UV-Vis) spectrophotometry is the most widely used method for detection of pigments, and by using a diode array detector (DAD), the entire absorption spectra can be recorded at once. Spectrophotometers typically give the intensity of a color at a certain wavelength as absorbance (A), a unit-less number that can be expressed though Lambert-Beers Law:

Eq. 6.1 $A = \log(I_0/I) = \varepsilon * l * c$ (Absorbance)

Lambert-Beers law states that the absorbance is equal to the logarithm of the ratio between I_0, the intensity of an unaffected beam of light and I, the intensity of the light after passing through the sample of interest. These values are more frequently expressed as the product of the sample concentration (c), the distance though the sample (l) and the compound specific constant ε, known as the extinction coefficient. Since the absorption is dependent on concentration, quantification of pigments is often based on UV-Vis measurements. The absorption maxima of MP ranges from 390 and 530 nm depending on solvent and pH (see Figure 2.3 on p. 10). Despite the usefulness of UV-Vis detection, the method has some limitations, as several MP have comparable absorption spectra (Jung et al., 2003) and cannot be clearly distinguished from each other. In these cases other detection techniques, such as mass spectrometry (MS), high resolution mass spectrometry (HRMS), or infrared (IR) spectrophotometry are needed in order to differentiate between very similar compounds. By combining several detection methods with separation strategies like HPLC, powerful means of detection can be achieved, such as HPLC-DAD-HRMS, giving information about retention time, chromophore and molecular composition simultaneously (Kildgaard et al., 2014; Mapari et al., 2006).

6.1.2 Quantitative assessment: isolation, purification and generation of standards

The most widely applied purification method for MP is liquid chromatography (LC) (Jung et al., 2003; Lin et al., 1992; Sun et al., 2005). Additionally, capillary electrophoresis (Watanabe et al., 1999; Watanabe & Yamamoto, 1997) and high-speed counter-current chromatography, have been reported as methods for purifying MP.

Some of the above mentioned detection techniques could also be used for pigment quantification. Based on the UV-Vis spectrum of individual pigments, standard curves can be used for pigment qualification using Lambert-Beers Law (Domenici et al., 2014; Said et al., 2014; P. B. Torres et al., 2014). Crucial for this method is a powerful purification strategy, which guarantees the presence of single pure pigments (99% pure). The use of MS and UV-Vis coupled to HPLC has also been reported for quantification (Fu et al., 2012).

6.2 Experimental strategy

All experiments for product identification were performed in shake flasks to prevent the contamination with antifoam. Firstly, major pigments in the fermentation broth from cultivation of *T. atroroseus* with KNO_3 as nitrogen source were identified via LC-MS and UV-Vis analysis. After identification of several potential novel compounds in the pigment cocktail of *T. atroroseus,* those were purified and their structure was confirmed by NMR- analysis. The experimental strategy can be found in Figure 6.3.

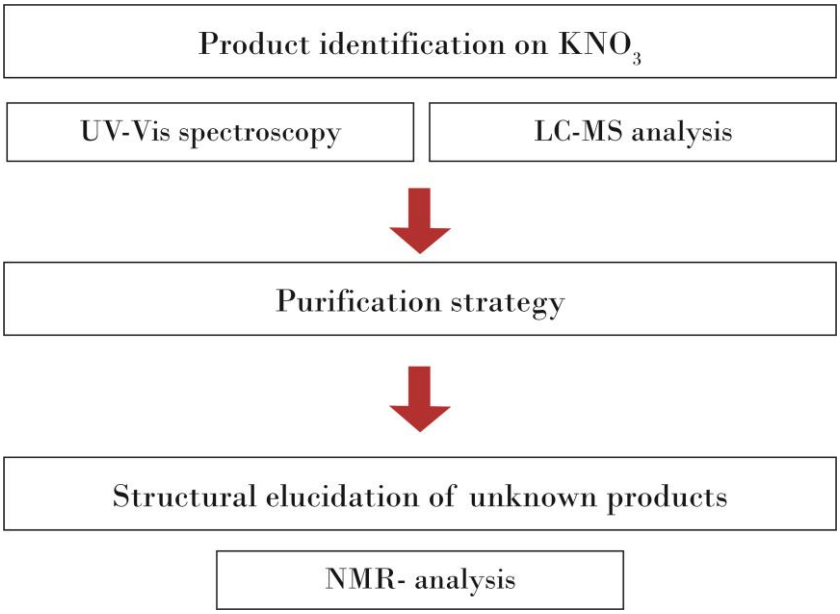


Figure 6.3 Experimental strategy for pigment identification of *T. atroroseus*

6.3 Material & Methods

A detailed description of spore preparation and sampling can be found in section 3 "**Materials & Methods**" (p. 29).

Trace metal solution (2 mL/L). The trace metal solution consisted of $CuSO_4 \cdot 5 H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10 H_2O$ (0.04 g/L), $FeSO_4 \cdot 7H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7H_2O$ (8 g/L).

Amino acid cultivation: The following medium was used: sucrose (7.5 g/L), glucose (0.375 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H_2O$ (0.1 g/L) and trace metal solution (2 mL/L). The nitrogen source was 0.1 M each of an L-amino acid. Controls were done using 0.1 M of KNO_3. The pH of the medium was adjusted to pH 5 with aqueous NaOH and HCl. Cultivations were carried out in non-baffled shake flasks at 30 °C and 150 rpm in rotary shaking incubators (Forma orbital shaker, Thermo Fisher Scientific, US) with a volume of 100 mL. Samples were taken after 96 hrs. Shake flask experiments were carried out in triplicates. And extractions were done on pooled samples after analysis.

Pigment yield was determined by measuring the absorbance at 500 nm and converting the value into g/L with the help of the standard curve from atrorosin-E shown in Figure 3.2 on p.37.

Purification and **Extraction** of atrorosins and PP-O was performed as described in section 3.11 "**Chemical analysis of the pigments**" on p. 34

A detailed description of **Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) analysis** and **1D and 2D NMR** analysis can also be found in section 3.11 "**Chemical analysis of the pigments**" on p. 34.

6.4 Results and Discussion

6.4.1 Red pigments on KNO_3

As described earlier, submerged cultivation of *T. atroroseus* with KNO_3 as nitrogen source resulted in an intensive red colored fermentation broth with absorbance maxima at 500 nm (see Figure 4.10 on p. 57). Analysis of the ethyl acetate extract of the fermentation liquid by Ultra High Performance Liquid Chromatography coupled to Diode Array Detection and High-Resolution tandem Mass Spectrometry (UHPLC-DAD-MS/HRMS) showed that a plethora of different red pigments was produced (Figure 6.4 A). Several known *Monascus* pigments (e.g. PP-R, PP-V and PP-Y) were identified in the fermentation broth along with the detection of numerous unknown pigments. Figure 6.4 A) shows an exemplary extracted UV-Vis chromatogram of the pigment mixture at 520 nm. Each major peak could be assigned a mass to charge ratio (m/z). Figure 6.4 B) illustrates the UV-Vis spectrum of the compound with m/z=500.1912. The strong absorption at 430 and 520 nm is similar to the absorption of known red MP.

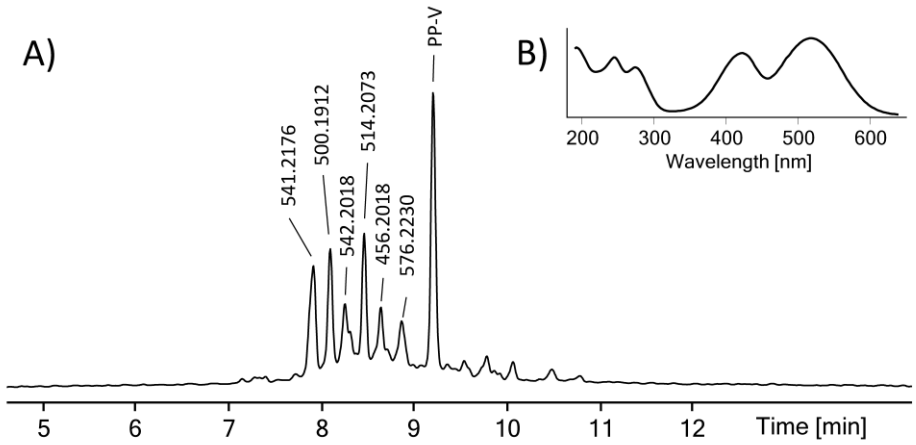


Figure 6.4 A) Extracted UV-Vis chromatogram (520±10nm) of the EtOAc extract from shake flask cultivation of *T. atroroseus* using KNO_3 as nitrogen source. The m/z or identity of the major peaks has been assigned according to the LC- MS data. B) UV-Vis spectrum of m/z=500.1912

6.4.2 Elucidation of atrorosin-S

The compound with m/z of 500.1912 was then purified by reverse phase chromatography to give a dark red amorphous solid. From HRMS the molecular

formula was determined to be $C_{26}H_{29}N_2O_9$ (DBE=13) (calc. 500.1915, mass accuracy = 0.6 ppm). Furthermore, 1D and 2D NMR was used to determine the structure of the compound. ^{1}H-NMR and HSQC revealed five alkene CH-groups, two methyl groups, seven CH_2-groups, and one alkane CH-group. ^{13}C-NMR and HMBC revealed an additional 11 quaternary carbons of which five were carbonyls, five alkene carbons, and one quaternary alkane. Additionally, two carboxylic acids and an amide were present in the molecule. Based on the structural information obtained from the NMR data, an isoquinoline system could be assembled as the core of the molecule, consisting of C-4 to C-12, with C-13 and C-14 forming a five-membered lactone with C-8 and C-9. Attached to the central ring structure, an α,β-unsaturated carboxylic acid and a methyl group could be linked to C-4 and C9, respectively. A heptanyl chain could be linked to C-14 via the ketone C-15. Finally, a serine moiety was determined to be attached through the isoquinoline nitrogen. The molecular structure is shown in Figure 6.5.

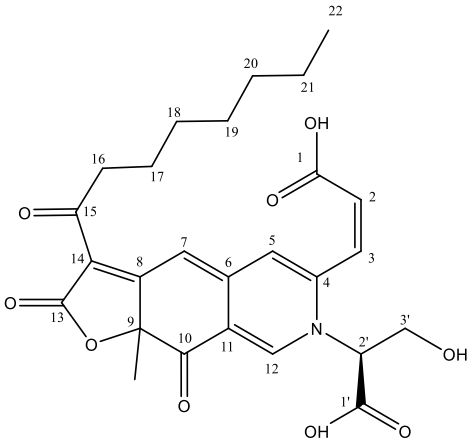


Figure 6.5 Numbered structure of atrorosin-S

The novel compound was structurally very similar to the known *Monascus* Pigments PP-O and PP-V (see Figure 2.5 on p. 16). Like PP-O and PP-V it contained a carboxylic acid. Additionally a serine moiety was incorporated into the azaphilone core. The compound was named atrorosin-S, using the one letter amino acid code to denote which nitrogen- containing compound has been incorporated.

The discovery of this novel azaphilone raised the question, as to whether *T. atroroseus* was able to incorporate other amino acids as well. Incorporation of amino acids into monascorubrin has previously been described in *Monascus* (C. Kim et al., 2006; Lin et al., 1992). The LC-MS data from the crude extract was re-examined and all tentative amino acid derived atrorosins except arginine, histidine,

lysine, methionine and proline could be identified in the chromatogram. However, purification of all these compounds was necessary to confirm their structure. Separation and purification of very similar compounds from the same cultivation is time-consuming and tedious and therefore a cultivation method was developed for production of essentially pure atrorosins by *T. atroroseus*. The method will be presented in detail in Chapter 7 " **Tailored pigment production in *T. atroroseus***" on p. 97.

6.4.3 Expansion of the atrorosin-catalogue

In order to confirm the hypothesis of amino acid derived atrorosins, *T. atroroseus* was cultivated in shake flasks using each of the remaining 19 amino acids as the sole nitrogen source. The ethyl acetate extract of the filtered culture liquid was analyzed by UHPLC-DAD-MS/HRMS for each of the 19 cultivations. For proline, no production of the corresponding atrorosins was neither expected nor detected, as the nitrogen atom in this amino acid is a secondary amine. So far, only incorporation of primary amines into the azaphilone core have been reported. For each of the other remaining amino acids, with the exception of tryptophan, the expected atrorosin was detected. UV-Vis chromatograms of the extracts are illustrated in Figure 6.6.

Surprisingly, cultivation with single amino acids as the sole nitrogen source, changed the secondary metabolite profile for *T. atroroseus* drastically. Nearly exclusively one single red compound was detected in the extract- the atrorosin corresponding to the fed amino acid. For only a few of the experiments (glycine, cysteine, lysine, threonine and tyrosine) also other colored compounds were produced in minor concentrations (Figure 6.6)

For tryptophan, the pigment production was either very low or completely absent. In the extract from the glycine cultivation, the concentration of atrorosin-G was very low. In the extract from the tyrosine cultivation significant amounts of both PP-Y and PP-O were found in addition to the expected atrorosin-Y. We speculate, that atrorosins are produced by amination of the precursor PP-O with an amino acids. The presence of PP-O and PP-Y in the tyrosine extract indicated incomplete incorporation of tyrosine. Tyrosine has a very low water solubility (0.4 g/L) which could explain the incomplete conversion.

Isolation and purification was only possible for seven more atrorosins (D, E, H, L, M, Q and T), based on the cultivation with amino acids as nitrogen source. The other missing atrorosins were semi-synthesized with PP-O as a precursor. More details on the synthesis with PP-O can be found in appendix C on p. 137.

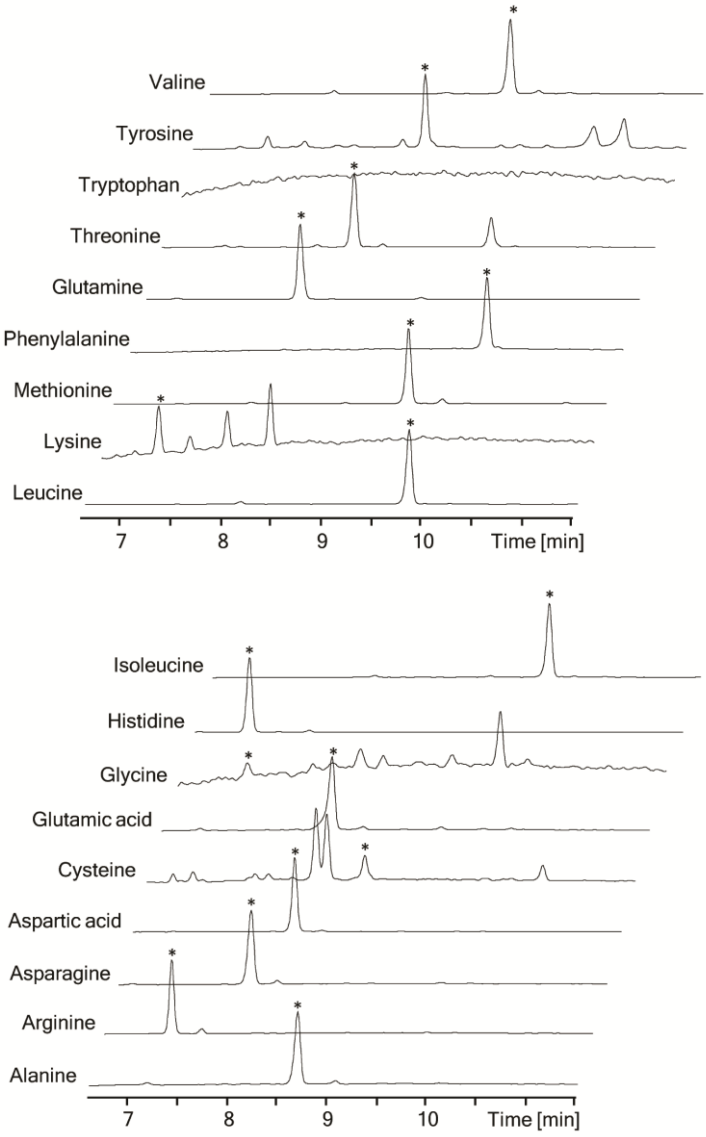
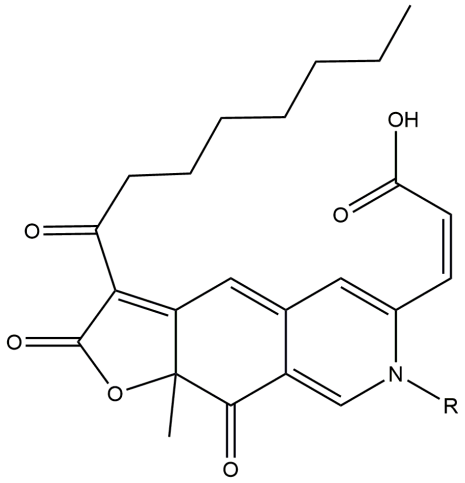


Figure 6.6 Extracted UV-Vis chromatograms (520±10 nm) of EtOAc extracts from shake flask cultivations of *T. atroroseus* using 18 different amino acids as sole nitrogen source. * indicates the expected atrorosins, confirmed by HRMS. Shake flasks cultivation were performed at 30 °C, at 150 rpm and a starting pH of 5.

The combination of amino-acid cultivation together with semi-synthesis from PP-O resulted in the identification of 19 novel atrorosins which are presented in Figure 6.7.

Figure 6.7 Overview of 19 novel atrorosins that were identified in *T.atroroseus*



R=	Ala:	Atrorosin-A
	Cys:	Atrorosin-C
	Asp:	Atrorosin-D
	Glu:	Atrorosin-E
	Phe:	Atrorosin-F
	Gly:	Atrorosin-G
	His:	Atrorosin-H
	Iso:	Atrorosin-I
	Lys:	Atrorosin-K
	Leu:	Atrorosin-L
	Met:	Atrorosin-M
	Asn:	Atrorosin-N
	Gln:	Atrorosin-Q
	Arg:	Atrorosin-R
	Ser:	Atrorosin-S
	Thr:	Atrorosin-T
	Val:	Atrorosin-V
	Trp:	Atrorosin-W
	Tyr:	Atrorosin-Y

6.5 Conclusion

T. atroroseus has been found to excrete high amounts of an entirely new class of red azaphilone pigments, denoted atrorosins. Atrorosins are derivatives of the orange pigment PP-O, differing only by incorporation of an amino acids. The amino acid moieties can be naturally incorporated during standard cultivation on KNO_3 yielding a plethora of different atrorosins. Amino acids can also be selectively incorporated by using single amino acids during cultivation as sole nitrogen source. This approach yields essentially only one atrorosin at a time and will be further described in Chapter 7 "**Tailored pigment production in *T. atroroseus***". Potentially, not only amino acids, but also other amino- group containing molecules like nucleotides or glucosamines (Hassan Hajjaj et al., 1997) could be incorporated into PP-O expanding the class of atrorosins even further.

Purification of PP-O and atrorosin-S allowed for the creation of standard curves that could be used for quantification of orange and red pigment in the fermentation medium. This standard curve was used to convert absorbance values detected in the scope of this project into concentrations in g/L - also retrospectively. Quantification of produced pigments is an essential step in the establishment of cell factory design because it allows concrete comparison of different processes. The standard curves of atrorosin-S and PP-O can be found in Figure 3.2. on p. 35 in the Material & Method section.

7 Tailored pigment production in *T. atroroseus*§

The last chapter of this thesis combines knowledge gained throughout the PhD project with regard to nitrogen source selection, process set-up and the pigment production mechanism of *T. atroroseus*. A high-yielding cultivation protocol for the selective production of individual atrorosins during submerged cultivation of *T. atroroseus* is presented and described.

§ This chapter is adapted from Tolborg *et al.* "Unique fermentation processes yielding pure azaphilones in *Talaromyces atroroseus"*. The manuscript was accepted in April 2018 by Microbial Cell Factories. Subsequently, submission was withdrawn to not be in conflict with a submitted patent application. The manuscript in its final form can be found in Appendix D on p.143.

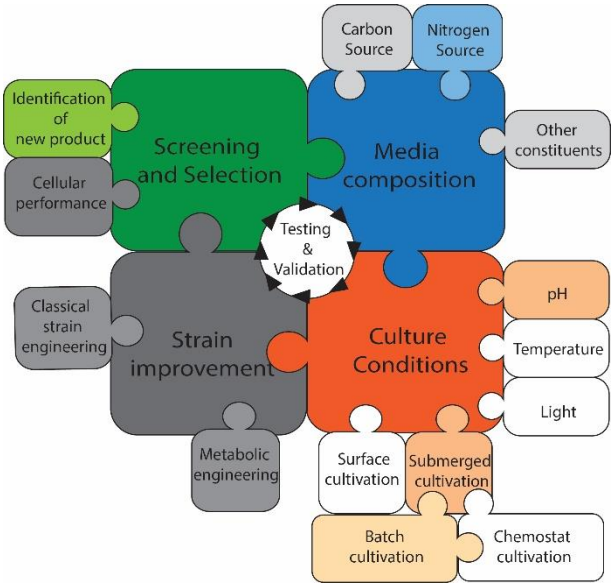


Figure 7.1 Iterative Process of implementing novel cell factories for pigment production; all parameters relevant for selective production of atrorosins are highlighted.

7.1 *Monascus* Pigment derivatives and their impact

In Asian countries, MP are used as food colorants under the name of "red rice" and their production process is well described (Ogihara & Oishi, 2002). Large-scale pigment production in *Monascus* and their industrial use, serve as an example for potential future applications of other fungal pigments.

One limitation of current production techniques of MP is that they result in the creation of mixtures of different yellow, orange and red constituents. Composition of these mixtures is difficult to control and the individual pigments are difficult to quantify (Sato, 1997). As already described in Chapter 4, MP biosynthesis is highly dependent on the medium conditions, and it was also shown in the literature, that different color shades can be produced through nitrogen source selection in *Monascus* (Shi et al., 2015). Generally, the color shift from yellow/orange compounds to red compounds is caused by the exchange of the oxygen in the pigment core by an amine. This reaction is thought to happen spontaneously and comprises ammonium, amino acids, amino sugars and proteins.

Feeding different amino-containing molecules to the fungus is thus a common strategy for expanding the catalogue of MP derivatives. For *Monascus* spp., supplementing ammonium nitrate with a specific amino acid lead to the incorporation of the amino acid into the pigment core structure and thus resulted in the formation of new pigment derivatives in the pigment cocktail (Jung et al., 2003; C. Kim et al., 2006).

The disadvantage of spontaneous amination is that the exact pigment composition is difficult to determine and control. Also batch to batch variations are expected. If fungal pigments aim for application in foods in Europe, standardized and controlled production is crucial.

To tailor pigment production in submerged cultivations, both the physiological conditions for optimal fungal growth as well as the conditions for pigment bio-synthesis need to be considered (Ogihara & Oishi, 2002). Media constituents including carbon and nitrogen sources as well as process parameters such as pH have shown to significantly affect pigment production in *T. atroroseus* (Chapter 4 "**Design of a cultivation medium supporting pigment production in *T. atroroseus***" on p. 39 and Chapter 5 "**Designing a cultivation process for enhanced pigment production in *T. atroroseus***" on p. 59). It was concluded, that optimal conditions for pigment production are not in accordance with optimal conditions for growth and that the pigment profile from *T. atroroseus* was influenced by the choice of nitrogen source since the production of different azaphilone pigments were observed on KNO_3 and $(NH_4)_2SO_4$. (section 4.4.6.1 "**Color profile of KNO_3 and $(NH_4)_2SO_4$**" on p. 56).

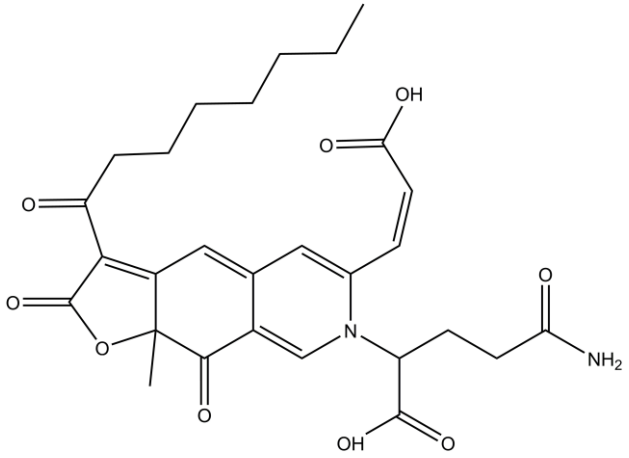


Figure 7.2 Structure of atrorosin-Q (identified by Rasmussen as N-glutamyl monascorubraminic acid (Rasmussen, 2015))

In 2015, Rasmussen identified a novel MP in *T. atroroseus* (Figure 7.2) and named it N-glutamyl monascorubraminic acid (Rasmussen, 2015). The compound contained a carboxylic acid moiety and the amino acid glutamine as incorporation in the azaphilone core. This indicated that also in *T. atroroseus*, incorporation of amino acids was possible. N-glutamyl monascorubraminic acid was later re-named atrorosin-Q (Isbrandt et al., n.d.). In 2017, on KNO_3 another novel pigment, was identified in the pigment portfolio of *T. atroroseus,* namely atrorosin-S. It was structurally similar to N-glutamyl monascorubraminic acid with the only difference being, that serine was incorporated in the pigment core instead of glutamine. Its discovery was described in Chapter 6. The discovery and elucidation of this novel atrorosins was based on a cultivation approach using only amino acids as nitrogen source (section 6.4.3 "**Expansion of the atrorosin-catalogue**" on p. 93). This cultivation strategy was the major driver in the discovery of the novel pigment class atrorosins in *T. atroroseus*. This discovery will be described and discussed in detail in the following chapter.

7.2 The experimental strategy

The aim of this chapter is to discuss the effect of amino acids on pigment biosynthesis in *T. atroroseus* IBT 11181.

Amino acids were screened in submerged shake flask cultivations as the sole nitrogen source with the aim to assess potential effects on in cellular performance and pigment production of *T. atroroseus*. Based on these results, two cultivation methods for production of single atrorosins have been developed. The experimental strategy can be found in Figure 7.3.

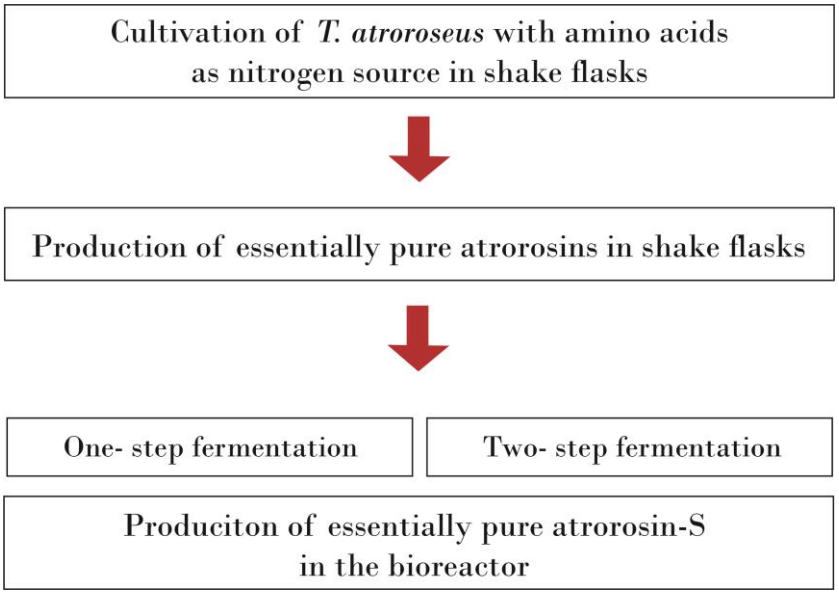


Figure 7.3 Experimental strategy for testing the effect of amino acids on pigment production in *T. atroroseus*

7.3 Material and Methods

A detailed description of spore preparation, bioreactor process details and sampling can be found in Chapter 4 "**Materials & Methods**" (p. 29).

Trace metal solution: The trace metal solution consisted of $CuSO_4 \cdot 5\ H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.04 g/L), $FeSO_4 \cdot 7\ H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2\ H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7\ H_2O$ (8 g/L).

Amino acid cultivation: The following medium was used: sucrose (7.5 g/L), glucose (0.375 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H_2O$ (0.1 g/L) and trace metal solution (2 mL/L). The nitrogen source was 0.1 M each of an L-amino acid. Controls were done using 0.1 M of KNO_3. The pH of the medium was adjusted to pH 5 with aqueous NaOH and HCl. Cultivations were carried out in non-baffled shake flasks at 30 °C and 150 rpm in rotary shaking incubators (Forma orbital shaker, Thermo Fisher Scientific, US) with a volume of 100 ml. Samples were taken after 96 hrs. Shake flask experiments were carried out in triplicates. And extractions were done on pooled samples after analysis.

Bioreactor cultivation on KNO_3: Submerged cultivation with KNO_3 as nitrogen source was carried out in a 1 L bioreactor. The medium recipe was: sucrose (7.5 g/L), glucose (0.375 g/L), KNO_3 (10 g/L) KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H_2O$ (0.1 g/L) and trace metal solution (2 mL/L).The cultivation was run at 30 °C, 800 rpm, 1 vvm and a pH of 4.5. The bioreactor experiments were carried out in duplicates.

Bioreactor cultivation on serine (one step): Submerged cultivation with serine as the sole nitrogen source was carried out in a 1 L bioreactor. For cultivation in bioreactors. 20 g/L sucrose and 10 g/L of serine were used. The other constituents were kept at the concentrations used in shake flasks. The cultivation was run at 30 °C, 800 rpm, 1 vvm and a pH of 4.5. The bioreactor experiments were carried out in duplicates.

Bioreactor cultivation with nitrogen- switch (two step): Two-step cultivations were carried out in 1 L bioreactors. The medium for two-step cultivation contained sucrose (20 g/L), glucose (1 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H2O$ (0.1 g/L) and trace metal solution (2 mL/L). For the first step, 2 g/L of KNO_3 were used as nitrogen source. After 53 h of cultivation the total concentration of PP-O in the medium was measured and a 10-fold concentration of serine was added (1 g/L) to induce formation of the amino acid derivative and to ensure optimal conversion. The cultivation was performed at 30 °C, 800 rpm, 1 vvm and a pH of 4.5.

Purification and **Extraction** of atrorosins and PP-O was performed as described in Section 3.11 "**Chemical analysis of the pigments**" on p. 34.

Pigment yield was determined by measuring the absorbance at 500 nm and converting the value into g/L with the help of the standard curve from atrorosin-E shown in Figure 3.2 on p.37.

7.4 Results and Discussion

As previously discussed, cultivation of *T. atroroseus* on KNO_3 resulted in the production of different red azaphilone pigments. The chromatogram illustrated in Figure 6.4 at page 91 allocates m/z ratios to the major peaks from the extracted UV-Vis chromatogram at 520±10 nm of the fermentation broth. After the discovery of 19 novel atrorosins, those peaks could be assigned to the respective pigment. The major pigments produced by *T. atroroseus* with KNO_3 as nitrogen source were PP-V (m/z = 411.1682), atrorosin-Q (m/z = 541.2176) atrorosin- S

(m/z = 500.1915), atrorosin-E (m/z = 542.2021) and atrorosin-T (m/z = 514.2072). This indicated that incorporation of ammonium (NH_4^+) as well as glutamine, glutamate, serine and threonine was favored over incorporation of other amino acids. On KNO_3 incorporation of the amino acids cannot be controlled and therefor the identification of the exact atrorosin composition was difficult, as some atrorosin derivatives were only produced in very small amounts. However, these results suggested that amino acids could be used as pre-cursor for the enhancement of atrorosin production in *T. atroroseus*.

7.4.1 Influence of amino acid as sole nitrogen source on biomass accumulation and pigment production

To assess the potential of all canonical amino acids as sole nitrogen source for biomass and pigment production, all 20 natural amino acids and ornithine were tested in shake flask experiments. A control containing KNO_3 as nitrogen source was also cultivated to benchmark biomass and pigment production. Final biomass values and final absorbance intensities for all 21 amino acids tested and the control are shown in Figure 7.4. All amino acids could be used for propagation of the fungus but only few amino acids promoted high pigment formation (aspartic acid, glutamate, serine).

Biomass accumulation was highest with glutamine (6.50±0.03 g/L), followed by proline (6.02±0.33 g/L), alanine (5.48±0.01 g/L) and ornithine (4.83±0.07 g/L). The use of arginine, asparagine, aspartic acid and glutamic acid gave similar results of round 4.5 g/L of biomass. The control with KNO_3 as nitrogen source yielded only 2.77±0.06 g/L biomass. Histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan and tyrosine had low biomass accumulation below 2 g/L.

As observed before, it wasn't necessarily the same amino acids that performed well in terms of biomass accumulation, that also promoted production of red pigments. There was no pigment production with proline and tryptophan, and very low absorbance intensities were measured when adding alanine and ornithine. Other low absorbance intensity (below 1) yielding amino acids were arginine, asparagine, cysteine, glutamic acid, glycine, isoleucine, lysine, methionine, phenylalanine, tyrosine and valine. The control with KNO_3 had an absorbance intensity of 0.68 AU/150µl. The highest absorbance intensity was obtained with glutamic acid (3.9 AU/150µl), followed by serine, aspartic acid and histidine. In contrast to glutamic acid, glutamine only yielded very low pigment concentration.

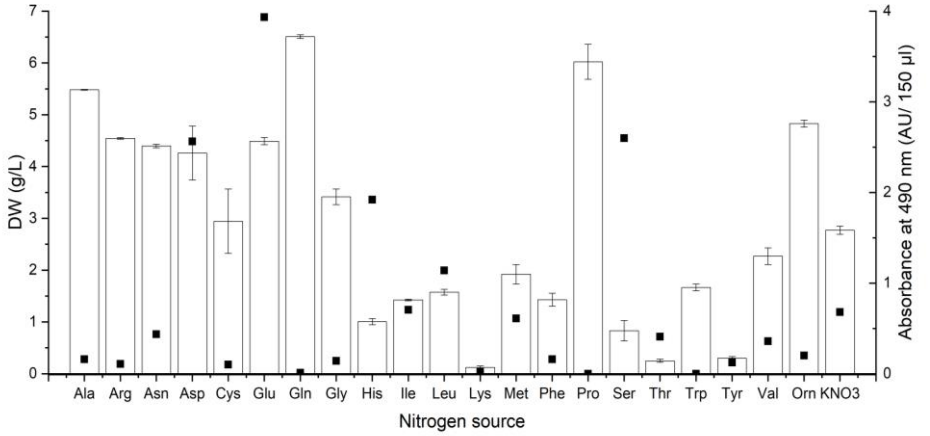


Figure 7.4 Biomass values (bar diagram) and absorbance intensity of fermentation broth (▪) of *T. atroroseus* cultures in shake flasks with single amino acids as sole nitrogen source after 96 h. Cultivation in shake flasks were performed at 30 °C, 150 rpm and at a starting pH of 5.

Product yields (Y_{SP}, absorbance intensity/ biomass) are shown in

Table 7.1 for the four amino acids with the highest product yields (serine, histidine, threonine, glutamic acid and the control KNO_3; other data can be found in Appendix E on p.177).

Even tough glutamate had the highest absolute pigment yield; the amino acid serine yielded the highest specific pigment production. This underlines once more that accumulation of a lot of biomass is not a strict requirement for a high pigment yield in *T. atroroseus*. Serine for instance only accumulated 1 g/L of biomass, but with an absorbance of 2.8 AU/150 µl it yielded the highest Y_{XP} of 3.1±0.02 Intensity (AU/150µl)/biomass (g/L) and seemed hence to be a promising fit as nitrogen source for pigment production in *T. atroroseus*. Serine, histidine, threonine and glutamate all had higher product yields (Y_{XP}) than using KNO_3.

The differences in pigment yields across the different amino acids could be caused by the dynamic environment in shake flasks. Shake flask cultivation do not allow for pH control, only offer suboptimal mixing and often suffer from limited oxygen saturation. The final pH of the cultures varied greatly, likely due to the different nature of amino acids, the way they were metabolized and their charge. pH values of the high yielding amino acids were around pH 4.1 and pH 5.7. Among all amino acids the pH varied from 3.0 to 5.7. As reported in section 5.4.3 "**pH study**" on p. 72 and in several other studies, pH is an important parameter for pigment biosynthesis (Carels, 1979; Carels & Shepherd, 1978; Méndez et al., 2011).

Table 7.1 Y_{XP} and final pH of the cultivation at 96 h of the four most promising amino acids as nitrogen sources and the control with KNO_3. The other data can be found in Appendix E on p 177

Nitrogen source	Yield Y_{XP}(Intensity (AU/150µl)/ biomass (g/L))	pH of liquid medium at the end of the cultivation
Ser	3.1 ±0.02	4.5 ± 0.13
His	1.9 ±0.06	4.2 ± 0.03
Thr	1.6 ±0.03	4.8 ± 0.06
Glu	0.9 ±0.07	5.7 ± 0.01
KNO_3	0.2 ±0.08	5.3± 0.03

7.4.2 LC-MS analysis of fermentation broth of shake flasks.

The five cultivation listed in

Table 7.1 where then analyzed via LC-MS. The corresponding extracted UV-Vis chromatograms are shown in Figure 7.5A). As seen previously, the already known mixture of atrorosins was produced when *T. atroroseus* was cultivated with KNO_3 as nitrogen source. The most abundant pigments were again PP-V and atrorosin derivatives from threonine, glutamine, serine and glutamate.

Interestingly, when a single amino acid was used as the sole nitrogen source, only one type of azaphilone pigment was produced, namely the respective atrorosin containing the fed amino acid.

Since only one or two peaks were present in each of the chromatograms, the purity of the fermentation broth with regard to red pigments was greatly increased compared to the control. The highest peak in the chromatogram of cultivation with serine, histidine, threonine and glutamate corresponded to the produced atrorosin. The smaller peak in the chromatograms of cultivation with serine, histidine and threonine corresponded to an amino-acid derivative without the carboxylic acid. Surprisingly, absorbance spectra of all amino acid derivatives were very similar with absorbance maxima at 245 nm, 275 nm, 420 nm and 520 nm. As an example, the absorbance spectrum from atrorosin-S is shown Figure 7.5.B).

That all amino acids lead to the same absorbance profile is in contrast to the work presented by Jung *et al.* 2003, who reported that in *Monascus*, amino acids like serine and glutamine would promote red color, while phenylalanine, isoleucine, leucine and valine would promote the production of orange and yellow pigments (Jung et al., 2003). We suspect that the orange color of their supernatant was observed due to incomplete incorporation of the respective amino acid into

the pigment structure, and were in fact owing to the presence of PP-O, monascorubrin, and other orange and yellow pigments in their samples. Jung *et al.* used a *Monascus* spp., which was cultivated with both, amino acids and low amounts of ammonium nitrate. Depletion of ammonium nitrate was not reported, suggesting that the orange color could have been caused by ammonium as the nitrogen source and not by the amino acid. Several studies in *Monascus* spp. demonstrated that the use of ammonium nitrate favors the formation of orange pigments. (Carels & Shepherd, 1977; Lin & Demain, 1995; Ogihara & Oishi, 2002).

By cultivation with amino acids as sole nitrogen source the formation of pigment mixture was avoided and only atrorosins, which all have a red chromophore, were produced.

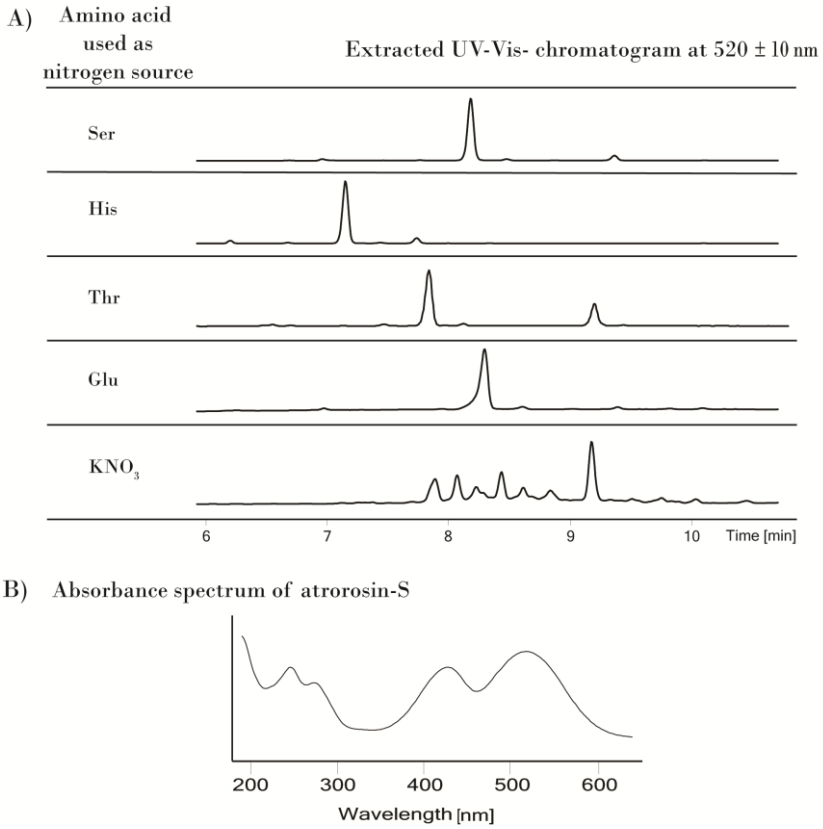


Figure 7.5 A) UV-Vis chromatograms (taken at 520±10 nm) of fermentation broth from cultivation with KNO_3 and different amino acids as nitrogen source. Shake flasks cultivation at 30 °C and 150 rpm. B) Exemplary absorbance spectrum of atrorosin-S from the same cultivation

7.4.3 Lab-scale amino acid-derivative production in the bioreactor

The high specific production yields and the superior pigment purity with amino acids as the sole nitrogen source was the basis for the following bioreactor cultivations. Serine was selected as nitrogen source since it yielded the highest specific pigment production in the shake flaks experiments. Cultivation in a bioreactor allows for pH control, controlled aeration and sparging which should increase both propagation and formation of pigments. Two cultivation methods were developed; one using only serine as the nitrogen source similar to the shake flask experiment (one-step) and one using a two-step procedure where KNO_3 is used as initial nitrogen source to allow for biomass accumulation followed by a nitrogen source switch to serine to induce pigment production. As shown in section 4.4.6.1" **Color profile of KNO_3 and $(NH_4)_2SO_4$"** on p. 56, pigment synthesis in *T. atroroseus* with KNO_3 as nitrogen source starts with the orange pigment PP-O. PP-O does not contain any nitrogen in the azaphilone core. It was also speculated in this thesis that PP-O production is promoted by nitrogen limitation (section 0 "**Chemostat with KNO_3 at pH 5"** on p. 81). This knowledge was applied in the two-step cultivation. First, PP-O production was promoted by inducing nitrogen-limited conditions and then, in a second step, with the addition of serine, PP-O was converted into atrorosin-S. A bioreactor cultivation with KNO_3 as nitrogen source served as a control. Exemplary cultivation plots from all three cultivation are shown in Figure 7.6. Cultivation A had 10 g/L KNO_3 as nitrogen source, cultivation B was the one-step cultivation and used 10 g/L serine as sole nitrogen source and cultivation C was the two-step cultivation and started with an initial concentration of 2 g/L KNO_3 and after depletion at approx. 55 h, 1 g/L of sterile serine was added to the bioreactor.

The cultivation with KNO_3 as nitrogen source was carried out with only 7.5 g/L sucrose. Yield coefficients will still be comparable with the one-step and the two-step cultivation, but the time course of the KNO_3 cultivation was in general shorter and had lower final titers due to the lower amount of initial carbon. Fungal propagation started at around 40 h and at 55 h carbon depletion was reached. CO_2 production also dropped at 55 h. However, DW accumulation and pigment production reached its maximum after 62 and 75 h. The cultivation yielded around 2.5 g/L of DW and 0.27 g/L of red pigment mixture. After carbon depletion at 55 h, the pigment detected in the fermentation broth changed slowly color from orange (PP-O) to red (atrorosins mixture) as seen in Figure 7.6 D). Just as

observed in the shake flask cultivation, a mixture of red pigments was produced (Figure 7.6 G).

Cultivation with serine as the sole nitrogen source reached carbon depletion at 180 h, yielding 0.9 g/L of atrorosin-S and 6.5 g/L of biomass (Figure 7.6 B). While cultivation duration was much longer compared to KNO_3, most of this can be attributed to a very long lag phase of almost 80 h. Atrorosin-S production increased with fungal growth during the entire time course of the fermentation and no PP-O production was observed (see Figure 7.6 E). It is noteworthy to state that there was minimal atrorosin impurity as can be seen in Figure 7.6 H).

Initial conditions of the two-step cultivation process included low amounts of KNO_3 (2 g/L), which allowed the fungus to propagate. In this process, the start of the cultivation was similar to the control with a short lag phase of 30 h before growth. The cultivation continued for 55 h producing essentially only the orange pigment PP-O shown in Figure 7.6 I). As indicated from the CO_2-production a limitation was reached at around 55 h, and CO_2-exhaust declined. Based on the low amounts of KNO_3 compared to the control, it was assumed that the nitrogen was depleted. At this time point, serine was added to the cultivation at a concentration of 1 g/L serving as new sole nitrogen source. Growth of the fungus continued and serine was incorporated into the PP-O azaphilone core structure, making the red pigment atrorosin-S as seen in Figure 7.6 F & I). At the end of the cultivation, all PP-O was converted into atrorosin-S as can be seen on the UV-Vis chromatogram in Figure 7.6 I). The two-step cultivation yielded 0.9 g/L of atrorosin-S, and 7.4 g/L biomass. Both, biomass accumulation and pigment production peaked at the time of carbon depletion at approx. 100h.

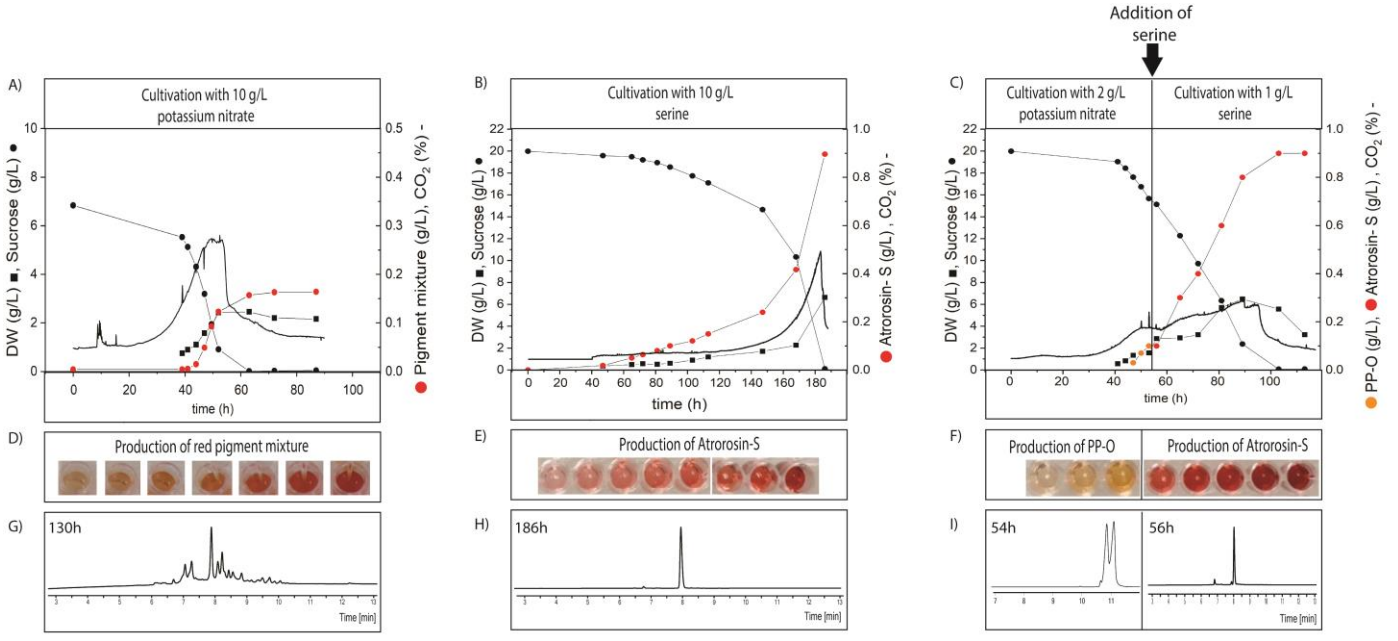


Figure 7.6 Time course of bioreactor cultivation with A) KNO_3, B) serine and C) with nitrogen source shift (two-step cultivation). Color profile of fermentation supernatant from cultivation with D) KNO_3, E) serine and F) of the two-step cultivation. UV-Vis chromatogram (520±10 nm) taken of the fermentation supernatant at the end of the cultivation from cultivation with G) KNO_3, H) serine and I) the two-step cultivation before and after the addition of serine. Bioreactor conditions: pH 4.5, 30 °C, 800 rpm.

Interestingly, careful analysis of the PP-O produced in the first step of the two-step cultivation showed the presence of what appeared to be a 3:2 ratio of two distinct isomers. These two isomers were isolated and separated using reverse phase HPLC to generate enough material for structural analysis. From NMR measurements, it was possible to identify the two PP-O isomers to be identical, except for having either a *cis*- or a *trans*- form of the double bond between C-2 and C-3. H-2 and H-3 had a coupling constant of J=12.8 Hz, establishing the alkene to be in a Z-configuration (Arai et al., 2015; Ogihara & Oishi, 2002). Since the *trans*-form of PP-O has not been reported before, this new compound was named *trans*-PP-O, and the known isomer will henceforth be referred to as *cis*-PP-O.

In the second step, where PP-O, was transformed into atrorosin- S, surprisingly both the *cis*- and *trans*-form of PP-O were converted to almost exclusively (>99.5%) the *cis* isomer of atrorosin-S (Figure 7.7). These results suggested that the amino acid incorporation into the core polyketide skeleton in *T. atroroseus* might be enzyme assisted. This theory has not been previously suggested, and could even involve transformation of the C-2/C-3 alkene in *trans*-PP-O into the *cis* form seen in atrorosins, since neither *trans*-PP-O nor "*trans*-atrorosins" could be detected after incorporation of amino acids.

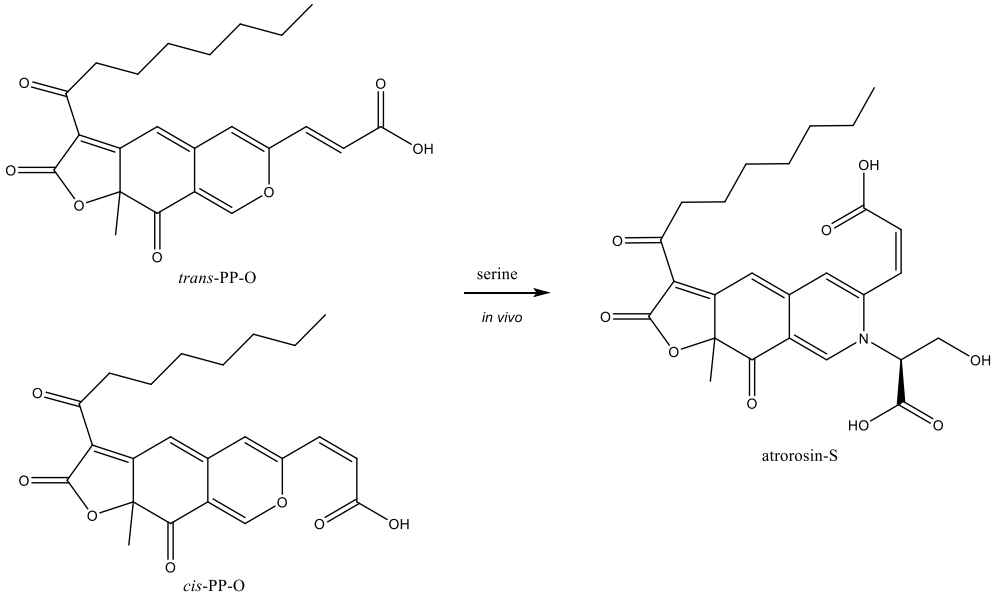


Figure 7.7 Schematic representation of the *cis*- and *trans*-form of PP-O and its conversion *in vivo* with Serine into *cis*-atrorosin-S.

Both the one-step and two-step cultivation yielded essentially pure *cis*-atrorosin-S. By introducing an initial growth step with KNO_3 as nitrogen source

(two-step), the cultivation time could be reduced to 50%, compared to when serine alone was used as a nitrogen source (one-step), while reaching the same final yield of *cis*-atrorosin-S.

7.4.4 Carbon to pigment conversion

Industrially it is relevant to evaluate the performance of the biomanufactoring processes based on the level of carbon converted into pigment. Carbon to pigment ratio (Y_{SP}) was calculated for the three bioreactor cultivations performed. Table 7.2 summarizes carbon conversion and evaluates pigment purity.

Table 7.2 Carbon to pigment conversion (in %) of cultivation on KNO_3, the one-step and the two-step cultivation

Nitrogen Source	KNO_3	One-step cultivation Serine	Two-step cultivation KNO_3 & Serine
Y_{SP} (c-mole/c-mole)	0.032	0.042	0.064
Carbon conversion to pigment	3.2 % Pigment mixture	4.2 % Atrororosin-S	6.4 % Atrororosin-S

Cultivations with KNO_3 produced a mixture of pigments, whereas the one-step and two-step only produces atrorosin-S. The total yield of pigment mixture from cultivation with KNO_3 was estimated using a standard curve of atrorosin-S for quantification. The use of the two-step process resulted in a 2-fold increase in carbon to pigment conversion compared to KNO_3, while also yielding essentially pure pigments.

When comparing the one-step and the two-step cultivation, the two-step seemed to be the preferred cultivation mode, as it had a shorter cultivation time and a better carbon-to-pigment conversion.

7.4.5 Potential pigment production mechanism for *T. atroroseus*

Based on the findings from the bioreactor cultivation on KNO_3, the one-step and the two-step fermentation, a potential scheme for pigment production in *T. atroroseus* was proposed and is shown in Figure 7.8.

A. At high concentrations of KNO_3, a mixture of atrorosins (ATR-X) and PP-V is produced. The high concentration of KNO_3 leads to excess of naturally biosynthesized amino acids in the fermentation broth. Those react with PP-O to form a plethora of atrorosins. For nitrate assimilation, KNO_3 is reduced to NH_4^+, which is thus also present in small amounts in the fermentation broth. This explains to formation of PP-V.

B. When serine is used as sole nitrogen source, essentially pure atrorosin-S is produced. Serine is in excess compared to other amino acids in the fermentation broth and is therefore preferred in the amination reaction of PP-O. This mechanism should also be true for other amino acids (shake flasks experiment).
C. At low concentrations of KNO_3, *T. atroroseus* experienced nitrate starvation and only produces PP-O. No amino acids are available for the amination reaction due to nitrogen starvation. *T. atroroseus* does not produce amino acids in excess but only those who are necessary for metabolism (3A). Once serine is added to the medium, the reaction of PP-O with serine results in essentially pure atrorosin-S (3B). This mechanism has also be validated with the amino acid glutamate (data not shown).

7.5 Conclusion

To assure optimal pigment production on different amino acids, cultivation of *T. atroroseus* in controlled bioreactors with pH control was essential.

Analysis of the produced pigments demonstrated that cultivation with individual amino acids as nitrogen sources in shake flasks resulted in production of their respective amino acid derivatives of PP-O. This cultivation approach did not only ease product recovery, but also resulted in higher specific pigment yields (Y_{XP}) compared to cultivation of *T. atroroseus* with KNO_3.

Furthermore, two methods for essential pure production of atrorosin-S in the bioreactor were presented. They resulted in significantly improved yields in regard to purity and quantity of atrorosin-S, and also had an improved carbon to pigment ratio compared to cultivation with KNO_3. Maximum Y_{SP} of 0.064±0.001 c-mole/c-mole was reached in the two-step cultivation.

Based on the finding in this chapter, a pigment production mechanism was proposed that explains the differences in atrorosin production in *T. atroroseus* depending on the nitrogen source used in the process.

Altogether, to the best of my knowledge, this chapter presents two novelties in regards to fungal cell factory design for pigment production.

1. Providing only one amino-containing molecule in excess to the fungal cell factory *T. atroroseus* (in this case an amino acid), it is for the first time possible to directly synthesize essentially pure pigments *in vivo*. By doing so, the problematic of mixed *Monascus* Pigments could be overcome.

2. The implementation of an high yielding atrorosin process aloowed for successful purification of individual atrorosins. Based on that, a standard curve could be created that made it possible to quantify pigments produced by *T. atroroseus*. So far in the literature most pigment yields are given in AU (see Table 2.2 on p. 26). This is the first time that actual product titers in g/L are reported for submerged production of MP.

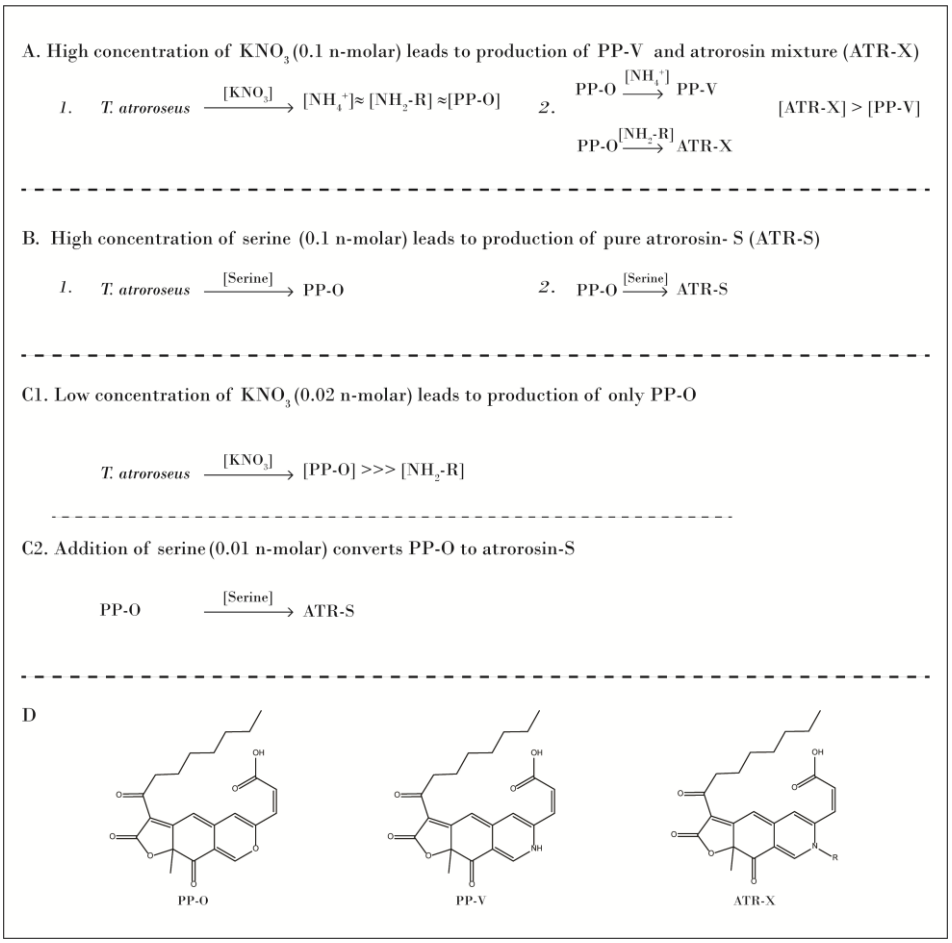


Figure 7.8 Proposed pigment production mechanism for *T. atroroseus* at different concentrations of KNO_3 and the amino acid serine, where ATR-X stands for mixture of atrorosins and ATR-S for atrorosin-S. D) Chemical structures of PP-O, PP-V and atrorosins

8 Conclusion and future perspective

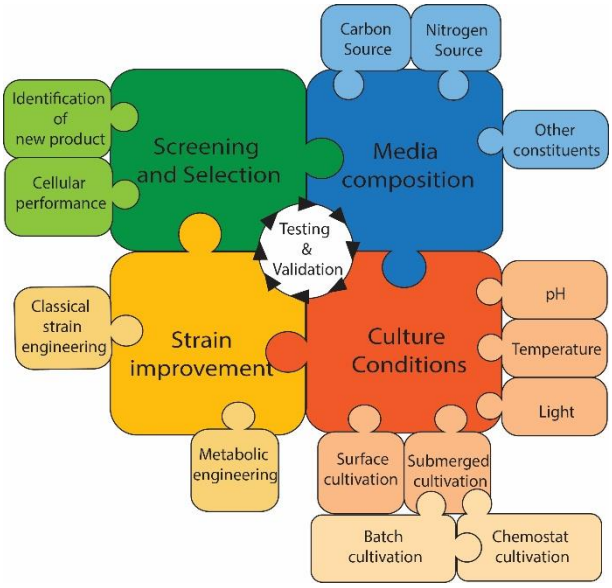


Figure 8.1 Iterative Process of implementing novel cell factories for pigment production.

Throughout this dissertation, important milestones towards the implementation of *T. atroroseus* as a fungal cell factory for red pigment production were achieved. Media and process conditions were found to heavily influence pigment production in *T. atroroseus* and a suitable cultivation medium as well as process conditions supporting pigment production were proposed. Known red *Monascus* Pigments could be identified in the pigment production portfolio of *T. atroroseus* and furthermore a novel class of azaphilone pigments was discovered and named atrorosins. Throughout this PhD project, the red pigment yield in *T .atroroseus* was improved by more than 400% (from Y_{SP} of 0.012 ± 0.001 c-mole/c-mole (**Chapter 4**) to Y_{SP} of 0.064 ± 0.001 c-mole/c-mole (**Chapter 7**))- by process design only and without the use of genetic engineering.

Standard reproducible cultivation protocols as well as development of tools for quantification and qualification of POI are key cornerstones in cell factory design (Figure 8.1).

This thesis compiles four result chapters which can be divided into media design (**Chapter 4**), process design (**Chapter 5**), discovery and characterization of atrorosins (**Chapter 6**) and tailored atrorosin production in *T. atroroseus* (**Chapter 7**). Conclusions summarizing the results and findings of the individual chapters can be found on p. 57 (**Chapter 4**), p. 84 (**Chapter 5**), p. 95 (**Chapter 6**) and p. 112 (**Chapter 7**).

Here, key findings across all chapters will be listed:

- Screening of different media compositions revealed highest pigment production of T. atroroseus with a combination of KNO3 and sucrose. A pigment production medium was proposed and validated in the bioreactor. Furthermore, the nitrogen source seemed to affect the kind of pigments produced by T. atroroseus.
- The cultivation mode did not seem to heavily influence pigment production as the same azaphilone pigments were produced both in submerged and surface cultivation of *T. atroroseus*. Investigation of how the pH affected cellular performance of *T. atroroseus* revealed major physiological differences at low (pH 3) and higher pH (pH 4.5). Pigment production was found to be best at pH 4.5, but under these conditions propagation of the fungus was hampered. A two-step fermentation process with a pH switch from pH 3 to pH 4.5 combined fungal growth at pH 3 with induction of

pigment production at pH 4.5 and could thereby increase the red pigment yield by 35%. Chemostat cultivation confirmed pH 3 to be favorable for cell growth and propagation. No red pigments were produced by *T. atroroseus* when maintained in continuous cultivation.

- The use of chemical analysis tools (LC-MS) allowed identification of different known *Monascus* Pigments (MP), including PP-O and PP-V in the pigment portfolio of *T. atroroseus*. NMR analysis of the most abundant unknown MP derivative in the fermentation broth of *T. atroroseus* led to the discovery of a group of novel fungal MP, named atrorosins. The term atrorosin describes *Monascus* Pigment derivatives of the known azaphilone pigment PP-O with different amino acids incorporated into the azaphilone core. Even though both *cis*- and *trans*- PP-O are precursors of atrorosins, atrorosins seemed practically only existent in *cis*-form.
- Product recovery could be greatly simplified by using single amino acids as sole nitrogen source in cultivation with *T. atroroseus*. Instead of the usual MP cocktail, essentially pure atrorosins were produced.
- Two fermentation set-ups for essentially pure atrorosin-S production were developed: the one-step process (only amino acid) and the two-step process (production of PP-O by inducing nitrate limiting conditions with KNO_3 and then transformation of PP-O into atrorosin by addition of single amino acid). Maximum pigment yields of 0.9 g/L were reached in the two-step process corresponding to a Y_{SP} of 0.064±0.001 c-mole/c-mole.
- Two requirements for pigment production in *T. atroroseus* could be defined: the pH had to be above pH4 and the nitrogen source had to be below a certain threshold (≈ 0.5 g/L)
- To the best of my knowledge, for the first time, production yields of MP could be given in g/L and not in AU. This could be realized by creation of a standard curve based on purified atrorosin-E.

8.1 Future perspectives

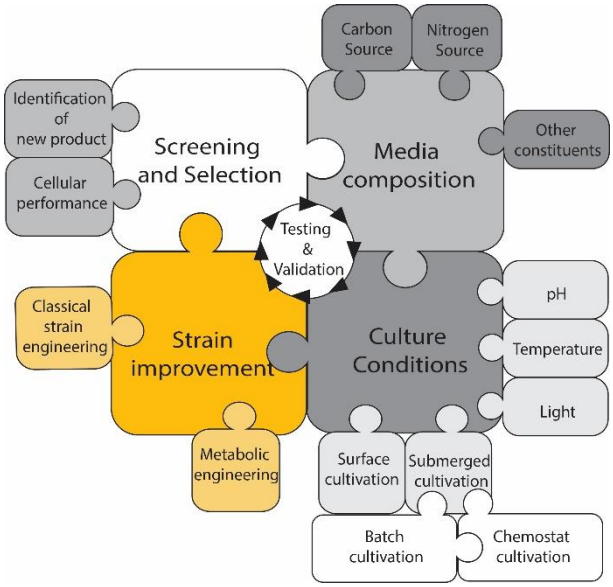


Figure 8.2 Iterative Process of implementing novel cell factories for pigment production; future perspectives are highlighted

After establishing reproducible high yielding process protocols and elucidating the pigment portfolio of *T. atroroseus*, the next step would consist in the remaining puzzle piece in the iterative process of implementing novel cell factories; strain improvement (see Figure 8.2).

Apart from improving physiological performance in submerged cultivation, genes directly involved in the biosynthesis of MP would here be obvious targets. Improvement of the pigment yield could be done by over-expression the transcriptional activator in the MP gene cluster, or by exchanging the promoter to a constitutive one.

However, for strain improvement as well as for further studies on biosynthesis of atrorosins, enhancement of the molecular toolbox will be of great importance. Especially the availability of a non-homologous-end-joining deficient strain of *T. atroroseus* would be of excellent use to improve the success rates in genetic transformation. In 2017, the CRISPR/ Cas9 system for effective genetic engineering has been successfully adapted in *T. atroroseus* and thereby paving the way for metabolic engineering strategies for pigment production in *T. atroroseus* (M. L. Nielsen et al., 2017).

9 Bibliography

Adrio, J. L., & Demain, A. L. **2003**. Fungal biotechnology. *Int. Microbiol.*, *6*(3), 191–199.

Ahn, J., Jung, J., Hyung, W., Haam, S., & Shin, C. **2006**. Enhancement of monascus pigment production by the culture of Monascus sp. J101 at low temperature. *Biotechnol. Prog.*, *22*(1), 338–340.

Akihisa, T., Tokuda, H., Yasukawa, K., Ukiya, M., Kiyota, A., Sakamoto, N., Suzuki, T., Tanabe, N., & Nishino, H. **2005**. Azaphilones, furanoisophthalides, and amino acids from the extracts of Monascus pilosus-fermented rice (red-mold rice) and their chemopreventive effects. *J. Agric. Food Chem.*, *53*(3), 562–565.

Akishina, R. I. **2001**. *WO2003038064 A8*.

Amchova, P., Kotolova, H., & Ruda-Kucerova, J. **2015**. Health safety issues of synthetic food colorants. *Regul. Toxicol. Pharmacol.*, *73*(3), 914–922.

Andersen, M. R., Lehmann, L., & Nielsen, J. **2009**. Systemic analysis of the response of Aspergillus niger to ambient pH. *Genome Biol.*, *10*(5), R47.

Arai, T., Koganei, K., Umemura, S., Kojima, R., Kato, J., Kasumi, T., & Ogihara, J. **2013**. Importance of the ammonia assimilation by Penicillium purpurogenum in amino derivative Monascus pigment, PP-V, production. *AMB Express*, *3*(1), 19.

Arai, T., Kojima, R., Motegi, Y., Kato, J., Kasumi, T., & Ogihara, J. **2015**. PP-O and PP-V, Monascus pigment homologues, production, and phylogenetic analysis in Penicillium purpurogenum. *Fungal Biol.*, *119*(12), 1226–1236.

Arai, T., Umemura, S., Ota, T., Ogihara, J., Kato, J., & Kasumi, T. **2012**. Effects of Inorganic Nitrogen Sources on the Production of PP-V [(10Z)-12-carboxyl-monascorubramine] and the Expression of the Nitrate Assimilation Gene Cluster by Penicillium sp. AZ. *Biosci. Biotechnol. Biochem.*, *76*(1), 120–124.

Arunachalam, C., & Narmadhapriya, D. **2011**. Monascus fermented rice and its beneficial aspects: A new review. *Asian J. Pharm. Clin. Res.*, *4*(1), 29–31.

Babitha, S., Soccol, C. R., & Pandey, A. **2007**a. Effect of stress on growth, pigment production and morphology of Monascus sp. in solid cultures. *J. Basic Microbiol.*, *47*(2), 118–126.

Babitha, S., Soccol, C. R., & Pandey, A. **2007**b. Solid-state fermentation for the production of Monascus pigments from jackfruit seed. *Bioresour. Technol.*, *98*(8), 1554–1560.

Balakrishnan, B., Chen, C. C., Pan, T. M., & Kwon, H. J. **2014**. Mpp7 controls regioselective Knoevenagel condensation during the biosynthesis of Monascus azaphilone pigments. *Tetrahedron Lett.*, *55*(9), 1640–1643.

Balakrishnan, B., Karki, S., Chiu, S. H., Kim, H. J., Suh, J. W., Nam, B., Yoon, Y. M., Chen, C. C., & Kwon, H. J. **2013**. Genetic localization and in vivo characterization of a Monascus azaphilone pigment biosynthetic gene cluster. *Appl. Microbiol. Biotechnol.*, *97*(14), 6337–6345.

Berg, J. M., Tymoczko, J. L., & Stryer, L. **2006**. *Biochemistry* (6th editio). W.H. Freeman and Company.

Berman, J., Zorrilla-Lopez, U., Farre, G., Zhu, C., Sandmann, G., Twyman, R. M., Capell, T., & Christou, P. **2014**. Nutritionally important carotenoids as consumer products. *Phytochem. Rev.*, *14*(5), 727–743.

Bianchi, A. **2005**. Extracts of Monascusus purpureus beyond statins-profile of efficacy and safety of the use of extracts of Monascus purpureus. *Chin J Integr Med*, *11*(4), 309–313.

Biesebeke, R. **2002**. Aspergillus oryzae in solid state and submerged fermentations progress report on a multi disciplinary project. *FEMS Yeast Res.*, *2*, 245–248.

Bijinu, B., Suh, J.-W., Park, S.-H., & Kwon, H.-J. **2014**. Delineating Monascus azaphilone pigment biosynthesis: oxidoreductive modifications determine the ring cyclization pattern in azaphilone biosynthesis. *RSC Adv.*, *4*(103), 59405–59408.

Blanc, P. J., Laussac, J. P., Le Bars, J., Loret, M. O., Pareilleux, A., Prome, D., Santerre, A. L., & Goma, G. **1995**. Characterization of monascidin A from Monascus as citrinin. *Int. J. Food Microbiol.*, *27*, 201–213.

Blanc, P. J., Loret, M. O., & Goma, G. **1995**. Production of citrinin by various speecies of monascus. *Biotechnol. Lett.*, *17*(3), 291–294.

Blanc, P. J., Loret, M. O., Santerre, A. L., Pareilleux, A., Prome, D., Prome, J. C., Laussac, J. P., & Goma, G. **1994**. Pigments of Monascus. *J. Food Sci.*, *59*(4), 862–865.

Brakhage, A. A. **1998**. Molecular regulation of beta-lactam biosynthesis in filamentous fungi. *Microbiol. Mol. Biol. Rev.*, *62*(3), 547–585.

Broder, C. U., & Koehler, P. E. **1980**. Pigments produced by Monascus purpureus with regard to quality and quantity. *J. Food Sci.*, *45*(3), 567–569.

Carels, M. **1979**. The effect of changes in pH on phosphate and potassium uptake by Monascus rubiginosus ATCC 16367 in submerged shaken culture. *Can. J. Microbiol.*, *25*.

Carels, M., & Shepherd, D. **1977**. The effect of different nitrogen sources on pigment

production and sporulation of Monascus species in submerged, shaken culture. *Can. J. Microbiol.*, *23*(10), 1360–1372.

Carels, M., & Shepherd, D. **1978**. The effect of pH and amino acids on conidiation and pigment production of Monascus major ATCC 16362 and Monascus rubiginosus ATCC 16367 in submerged shaken culture. *Can. J. Microbiol.*, *24*(11), 1346–1357.

Carvalho, J. C., Oishi, B. O., Woiciechowski, A. L., Pandey, A., Babitha, S., & Soccol, C. **2007**. Effect of substrates on the production of Monascus biopigments by solid-state fermentation and pigment extraction using different solvents. *Indian J. Biotechnol.*, *6*(2), 194–199.

Chen, D., Xue, Y., Chen, M., Li, Z., & Wang, C. **2016**. Optimization of submerged fermentation medium for citrinin-free monascin production by Monascus. *Prep. Biochem. Biotechnol.*, *6068*(April).

Chen, F. C. **1969**. The structure of monascin. *J. Chem. Soc. D Chem. Commun.*, (3), 130.

Chen, M., & Johns, M. **1993**. Effect of pH and nitrogen source on pigment production by Monascus purpureus. *Appl. Microbiol. Biotechnol.*, 132–138.

Chen, W., Chen, R., Liu, Q., He, Y., He, K., Ding, X., Kang, L., Guo, X., Xie, N., Zhou, Y., Lu, Y., Cox, R. J., Molnár, I., Li, M., Shao, Y., & Chen, F. **2017**. Orange, red, yellow: biosynthesis of azaphilone pigments in Monascus fungi. *Chem. Sci.*, *8*(7), 4917–4925.

Christensen, L. H., & Henriksen, C. M. **1995**. Continuous cultivation of Penicillium chrysogenum. Growth on glucose and penicillin production. *J. Biotechnol.*, *42*, 95–107.

Consumer Reports® national Research Center. **2015**. *Natural food Labels Survey*.

Davison, J., al Fahad, A., Cai, M., Song, Z., Yehia, S. Y., Lazarus, C. M., Bailey, a. M., Simpson, T. J., & Cox, R. J. **2012**. Genetic, molecular, and biochemical basis of fungal tropolone biosynthesis. *Proc. Natl. Acad. Sci.*, *109*(20), 7642–7647.

de Carvalho, J. C., Oishi, B. O., Pandey, A., & Soccol, C. R. **2005**. Biopigments from Monascus: Strains selection, citrinin production and color stability. *Brazilian Arch. Biol. Technol.*, *48*(6), 885–894.

Delgado-vargas, F. **2003**. Natural Colorants for Food and Nutraceutical Uses.

Domenici, V., Ancora, D., Cifelli, M., Serani, A., Veracini, C. A., & Zandomeneghi, M. **2014**. Extraction of pigment information from near-UV vis absorption spectra of extra virgin olive oils. *J. Agric. Food Chem.*, *62*(38), 9317–9325.

Downham, A., & Collins, P. **2000**. Colouring our foods in the last and next millennium. *Int. J. Food Sci. Technol.*, *35*(1), 5–22.

Dufossé, L. **2006**. Microbial production of food grade pigments. *Food Technol. Biotechnol.*, *44*(3), 313–321.

Dufossé, L., Fouillaud, M., Caro, Y., Mapari, S. A. S., & Sutthiwong, N. **2014**.

Filamentous fungi are large-scale producers of pigments and colorants for the food industry. *Curr. Opin. Biotechnol.*, *26C*, 56–61.

Dufossé, L., Galaup, P., Yaron, A., Arad, S. M., Blanc, P., Chidambara Murthy, K., & Ravishankar, G. **2005**. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends Food Sci. Technol.*, *16*(9), 389–406.

E. Sardaryan. **2002**. *US 6340586 B1*.

Feng, Y., Shao, Y., & Chen, F. **2012**. Monascus pigments. *Appl. Microbiol. Biotechnol.*, *96*(6), 1421–1440.

Fielding, B. C., Haws, E. J., Holker, J. S. E., Powell, A. D. G., Robertson, A., Stanway, D. N., & Whalley, W. B. **1960**. Monascorubrin. *Tetrahedron Lett.*, *1007*(5), 24–27.

Firn, R. D., & Jones, C. G. **2003**. Natural products - a simple model to explain chemical diversity. *Nat. Prod. Rep.*, *20*(4), 382–391.

Frisvad, J. C., Yilmaz, N., Thrane, U., Rasmussen, K. B., Houbraken, J., & Samson, R. **2013**. Talaromyces atroroseus, a New Species Efficiently Producing Industrially Relevant Red Pigments. *PLoS One*, *8*(12), e84102.

Fu, W., Magnúsdóttir, M., Brynjólfson, S., Palsson, B., & Paglia, G. **2012**. UPLC-UV-MSE analysis for quantification and identification of major carotenoid and chlorophyll species in algae. *Anal. Bioanal. Chem.*, *404*(10), 3145–3154.

Gao, J., Yang, S., & Qin, J. **2013**. Azaphilones: Chemistry and Biology. *Chem. Rev.*, *113*(7), 4755–4811.

Gessler, N. N., Egorova, A. S., & Belozerskaya, T. A. **2013**. Fungal anthraquinones. *Appl. Biochem. Microbiol.*, *49*(2), 85–99.

Gheith, O., Sheashaa, H., & Abdelsalam, M. **2008**. Efficacy and safety of Monascus purpureus Went rice in children and young adults with secondary hyperlipidemia: A preliminary report. *Eur. J. Intern. Med.*, *20*(3), 57–61.

Gmoser, R., Ferreira, J. A., Lennartsson, P. R., & Taherzadeh, M. J. **2017**. Filamentous ascomycetes fungi as a source of natural pigments. *Fungal Biol. Biotechnol.*, *4*(1), 4.

Grand view Research. **2014**. Food Colorants Market Analysis By Product (Synthetic, Natural, Natural Identical, Caramel), By Application (Food, CSDs, Non Alcoholic Beverages, Alcoholic Beverages) And Segment Forecasts To 2020. Retrieved from http://www.grandviewresearch.com/industry-analysis/food-colorants-market

Grand view Research. **2018**. *Natural Food Colors Market Market, by product (Carotenoids, Curcumin, Anthocyanins, Carmine, Copper Chlorophyllin) Market, by application (Bakery & Confectionery, Beverages, Dairy & Frozen Products, Meat Products, Oil & Fat, Fruits & Vegetables, Food).*

Gunasekaran, S., & Poorniammal, R. **2008**. Optimization of fermentation conditions for red pigment production from Penicillium sp . under submerged cultivation.

African J. Biotechnol., *7*(12), 1894–1898.

Hajjaj, H., Blanc, P., Groussac, E., Goma, G., Uribelarrea, J., & Loubiere, P. **1999**. Improvement of red pigment/citrinin production ratio as a function of environmental conditions by monascus ruber. *Biotechnol. Bioeng.*, *64*(4), 497–501.

Hajjaj, H., Klaébé, A., Loret, M. O., Goma, G., Blanc, P. J., & François, J. **1999**. Biosynthetic Pathway of Citrinin in the Filamentous Fungus Monascus ruber as Revealed by 13C Nuclear Magnetic Resonance. *Appl. Environ. Microbiol.*, *65*(1), 311–314.

Hajjaj, H., Klaébé, A., Loret, M. O., Tzédakis, T., Goma, G., & Blanc, P. J. **1997**. Production and identification of N-glucosylrubropunctamine and N-glucosylmonascorubramine from Monascus ruber and occurrence of electron donor-acceptor complexes in these red pigments. *Appl. Environ. Microbiol.*, *63*(7), 2671–2678.

Harmsen, G. W., & Kolff, W. T. **1947**. Cultivation of Microorganisms with the Aid of Cellophane. *Science (80-.).*, *105*(2735), 582–583.

Haws, E. J., Holker, J. S. E., Kelly, A., Powell, A. D. G., & Robertson, A. **1959**. The Chemistry of fungi.Part XXXVII. The Structure of Rubropunctatin. *J Chem Soc*, *70*, 3598–3610.

He, Y., Liu, Q., Shao, Y., & Chen, F. **2013**. Ku70 and ku80 null mutants improve the gene targeting frequency in Monascus ruber M7. *Appl. Microbiol. Biotechnol.*, *97*(11), 4965–4976.

Hsu, W. H., & Pan, T. M. **2014**. Treatment of metabolic syndrome with ankaflavin, a secondary metabolite isolated from the edible fungus Monascus spp. *Appl. Microbiol. Biotechnol.*, *98*(11), 4853–4863.

Hu, Z., Zhang, X., Wu, Z., Qi, H., & Wang, Z. **2012**. Perstraction of intracellular pigments by submerged cultivation of Monascus in nonionic surfactant micelle aqueous solution. *Appl. Microbiol. Biotechnol.*, *94*(1), 81–89.

Hubert, J., Nuzillard, J. M., & Renault, J. H. **2017**. Dereplication strategies in natural product research: How many tools and methodologies behind the same concept? *Phytochem. Rev.*, *16*(1), 55–95.

Isbrandt, T., Tolborg, G., Workman, M., & Larsen, T. O. (n.d.). Atrorosins: A new subgroup of Monascus pigments from Taleromyces atroroseus.

James, T. W. **1961**. Continuous culture of microorganisms. *Annu. Rev. Microbiol.*, *15*(1), 27–46.

Jang, H., Choe, D., & Shin, C. S. **2014**. Novel derivatives of monascus pigment having a high CETP inhibitory activity. *Nat. Prod. Res.*, *28*(18), 1427–1431.

Jo, D., Deokyeong, C., Nam, K., & Shin, C. S. **2014**. Biological evaluation of novel derivatives of the orange pigments from Monascus sp. as inhibitors of melanogenesis. *Biotechnol. Lett.*, *36*(8), 1605–1613.

Joardar, V., Abrams, N. F., Hostetler, J., Paukstelis, P. J., Pakala, S., Pakala, S. B.,

Zafar, N., Abolude, O. O., Payne, G., Andrianopoulos, A., Denning, D. W., & Nierman, W. C. **2012**. Sequencing of mitochondrial genomes of nine Aspergillus and Penicillium species identifies mobile introns and accessory genes as main sources of genome size variability. *BMC Genomics*, *13*(1), 698.

John Villadsen, Jens Nielsen, G. L. **2011**. *Bioreaction Engineering Principles* (Third Edid). Springer Berlin Heidelberg.

Johns, M. R., & Stuart, D. M. **1991**. Production of pigments by Monascus purpureus in solid culture. *J. Ind. Microbiol.*, *2007*, 23–28.

Joshi, V. K., Attri, D., Baja, A., & Bhushan, S. **2003**. Microbial Pigments. *Indian J. Biotechnol.*, *2*(July), 362–369.

Jung, H., Kim, C., Kim, K., & Shin, C. S. **2003**. Color characteristics of monascus pigments derived by fermentation with various amino acids. *J. Agric. Food Chem.*, *51*(5), 1302–1306.

Jung, H., Kim, C., & Shin, C. S. **2005**. Enhanced photostability of monascus pigments derived with various amino acids via fermentation. *J. Agric. Food Chem.*, *53*(18), 7108–7114.

Juzlova, P., Martinkova, L., & Kfen, V. **1996**. Secondary metabolites of the fungus Monascus : a review. *J. Ind. Microbiol. Biotechnol.*, *16*, 163–170.

Kang, B., Zhang, X., Wu, Z., Wang, Z., & Park, S. **2014**. Production of citrinin-free Monascus pigments by submerged culture at low pH. *Enzyme Microb. Technol.*, *55*, 50–57.

Keller, N. P., Turner, G., & Bennett, J. W. **2005**. Fungal secondary metabolism - from biochemistry to genomics. *Nat Rev Microbiol*, *3*(12), 937–947.

Kildgaard, S., Mansson, M., Dosen, I., Klitgaard, A., Frisvad, J. C., Larsen, T. O., & Nielsen, K. F. **2014**. Accurate dereplication of bioactive secondary metabolites from marine-derived fungi by UHPLC-DAD-QTOFMS and a MS/HRMS library. *Mar. Drugs*, *12*(6), 3681–3705.

Kim, C., Jung, H., Kim, Y. O., & Shin, C. S. **2006**. Antimicrobial activities of amino acid derivatives of monascus pigments. *FEMS Microbiol. Lett.*, *264*(1), 117–124.

Kim, H. Y., Heo, D. Y., Park, H. M., Singh, D., & Lee, C. H. **2016**. Metabolomic and transcriptomic comparison of solid-state and submerged fermentation of Penicillium Expansum KACC 40815. *PLoS One*, *11*(2), 1–14.

King, T. **1973**. Studies in Mycological Chemistry. Part XXX and Last. Isolation and Structure of Purpuride, a Metabolite of Penicillium purpurogenum Stoll, 78–80.

Knight, R. D., Jones, B., & Field, S. **2007**. *College physics : a strategic approach*. San Francisco: Pearson/Addison Wesley.

Knudsen, P. B. **2015**. *Development of scalable high throughput fermentation approaches for physiological characterisation of yeast and filamentous fungi*. Technical Univeristy of Denmark.

Kobylewski, S., & Jacobson, M. F. **2010**. Food Dyes: A Rainbow of Risks. *Decis. Sci.*, *30*(2), 337–360.

Koehler, B. P. E. **1980**. Pigments produced by Monascus purpureus with regard to quality and quantity. *J. F*, *45*(1973), 567–569.

Kojima, R., Arai, T., Matsufuji, H., Kasumi, T., Watanabe, T., & Ogihara, J. **2016**. The relationship between the violet pigment PP-V production and intracellular ammonium level in Penicillium purpurogenum. *AMB Express*, *6*(1), 43.

Kongruang, S. **2011**. Growth kinetics of biopigment production by Thai isolated Monascus purpureus in a stirred tank bioreactor. *J. Ind. Microbiol. Biotechnol.*, *38*(1), 93–99.

Krairak, S., Yamamura, K., Irie, R., Nakajima, M., Shimizu, H., Chim-Anage, P., Yongsmith, B., & Shioya, S. **2000**. Maximizing yellow pigment production in fed-batch culture of Monascus sp. *J. Biosci. Bioeng.*, *90*(4), 363–367.

Kumasaki, S., & Nakanishi, K. **1962**. Structure of monascorubrin. *Tetrahedron*, *18*(5), 1171–1184.

Lampila, L. E. I., Wallen, S. E., Bullerman, L. B., Science, F., & Hall, F. **1985**. A review o f factors affecting biosynthesis of carotenoids by the order Monascus. *Mycopathologia*, *80*, 65–80.

Lin, T. F., & Demain, A. L. **1991**. Effect of nutrition of Monascus sp. on formation of red pigments. *Appl Microbiol Biotechnol*, *36*, 70–75.

Lin, T. F., & Demain, A. L. **1993**. Resting cell studies on formation of water-soluble red pigments by Monascus sp . *J. Ind. Microbiol.*, *12*, 361–367.

Lin, T. F., & Demain, A. L. **1994**. Leucine interference in the produc-tion of water-soluble red Monascus pigments. *Arch Microbiol*, *162*, 114–119.

Lin, T. F., & Demain, A. L. **1995**. Negative effect of ammonium nitrate as nitrogen source on the production of water-soluble red pigments by Monascus sp. *Appl Microbiol Biotechnol*, *43*, 701–705.

Lin, T. F., Yakushijin, K., Büchi, G. H., & Demain, A. L. **1992**. Formation of water-soluble Monascus red pigments by biological and semi-synthetic processes. *J. Ind. Microbiol.*, *9*(3–4), 173–179.

Liu, D. C., Wu, S. W., & Tan, F. J. **2010**. Effects of addition of anka rice on the qualities of low-nitrite Chinese sausages. *Food Chem.*, *118*(2), 245–250.

López-Nieto, M. J., Costa, J., Peiro, E., Méndez, E., Rodríguez-Sáiz, M., De La Fuente, J. L., Cabri, W., & Barredo, J. L. **2004**. Biotechnological lycopene production by mated fermentation of Blakeslea trispora. *Appl. Microbiol. Biotechnol.*, *66*(2), 153–159.

Lubertozzi, D., & Keasling, J. D. **2009**. Developing Aspergillus as a host for heterologous expression. *Biotechnol. Adv.*, *27*(1), 53–75.

Manchard, P. S., & Whalley, W. B. **1973**. Isolation and Structure of ankaflavin: a new

pigment from Monascus Anka. *Phytochemistry*, *12*(1963), 2531–2532.

Mapari, S. A. S. **2007**. *EP 2262862 A2*.

Mapari, S. A. S. **2008**. *Chemotaxonomic Exploration of Fungal Biodiversity for Polyketide Natural Food Colorants*. Technical University of Denmark.

Mapari, S. A. S., Hansen, M. E., Meyer, A. S., & Thrane, U. **2008**. Computerized screening for novel producers of Monascus-like food pigments in Penicillium species. *J. Agric. Food Chem.*, *56*(21), 9981–9989.

Mapari, S. A. S., Meyer, A. S., & Thrane, U. **2009**. Photostability of natural orange-red and yellow fungal pigments in liquid food model systems. *J. Agric. Food Chem.*, *57*(14), 6253–6261.

Mapari, S. A. S., Meyer, A. S., Thrane, U., & Frisvad, J. C. **2009**. Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale. *Microb. Cell Fact.*, *8*, 24.

Mapari, S. A. S., Meyer, A., & Thrane, U. **2006**. Colorimetric characterization for comparative analysis of fungal pigments and natural food colorants. *J. Agric. Food Chem.*, *54*(19), 7027–7035.

Mapari, S. A. S., Nielsen, K. F., Larsen, T. O., Frisvad, J. C., Meyer, A. S., & Thrane, U. **2005**. Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants. *Curr. Opin. Biotechnol.*, *16*(2), 231–238.

Mapari, S. A. S., Thrane, U., & Meyer, A. S. **2010**. Fungal polyketide azaphilone pigments as future natural food colorants? *Trends Biotechnol.*, *28*(6), 300–307.

Martin, J. F., & Demain, A. L. **1980**. Control of antibiotic biosynthesis. *Microbiol. Rev.*, *44*(2), 230–251.

Marzluf, G. A. **1997**. Genetic regulation of nitrogen metabolism in the fungi. *Microbiol. Mol. Biol. Rev.*, *61*(1), 17–32.

Medentsev, A. G., & Akimenko, V. K. **1998**. Naphthoquinone metabolites of the Fungi. *Phytochemistry*, *47*(6), 935–959.

Medentsev, A. G., Arinbasarova, A. Y., & Akimenko, V. K. **2005**. Biosynthesis of naphthoquinone pigments by fungi of the genus Fusarium. *Appl. Biochem. Microbiol.*, *41*(5), 503–507.

Méndez, A., Pérez, C., Montañéz, J. C., Martínez, G., & Aguilar, C. N. **2011**. Red pigment production by Penicillium purpurogenum GH2 is influenced by pH and temperature. *Biomed & Biotechnol*, *12*(12), 961–968.

Mohamed, M. S., Mohamad, R., Manan, M. A., & Ariff, A. B. **2012**. Enhancement of Red Pigment Production by Monascus purpureus FTC 5391 through Retrofitting of Helical Ribbon Impeller in Stirred-Tank Fermenter. *Food Bioprocess Technol.*, *5*(1), 80–91.

Mohan Kumari, H. P., Akhilender Naidu, K., Vishwanatha, S., Narasimhamurthy, K.,

& Vijayalakshmi, G. **2009**. Safety evaluation of Monascus purpureus red mould rice in albino rats. *Food Chem. Toxicol.*, *47*(8), 1739–1746.

Mørkeberg, R., Carlsen, M., & Nielsen, J. **1995**. Induction and repression of alpha-amylase production in batch and continuous cultures of Aspergillus oryzae. *Microbiology*, *141 (Pt 1*(1 995), 2449–2454.

Morton, A. G., & MacMillan, A. **1953**. The Assimilation of Nitrogen from Ammonium Salts and Nitrate by Fungi. *J. Exp. Bot.*, *5*(14), 232–252.

Nielsen, J. **2006**. Microbial process kinetics. In *Basic Biotechnology* (3rd ed.). Cambridge University Press.

Nielsen, K. F., Månsson, M., Rank, C., Frisvad, J. C., & Larsen, T. O. **2011**. Dereplication of microbial natural products by LC-DAD-TOFMS. *J. Nat. Prod.*, *74*(11), 2338–2348.

Nielsen, M. L., Isbrandt, T., Rasmussen, K. B., Thrane, U., Hoof, J. B., Larsen, T. O., & Mortensen, U. H. **2017**. Genes Linked to Production of Secondary Metabolites in Talaromyces atroroseus Revealed Using CRISPR-Cas9. *PLoS One*, *12*(1), e0169712.

Nødvig, C. S., Nielsen, J. B., Kogle, M. E., & Mortensen, U. H. **2015**. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One*, *10*(7), 1–18.

Novick, A., & Szilard, L. **1950**. Description of the Chemostat. *Science (80-.).*, *112*(2920), 715–716.

Ogihara, J., Kato, J., Oishi, K., & Fujimoto, Y. **2000**. Biosynthesis of PP-V, a Monascorubramine Penicillium sp. AZ Homologue, by Penicillium sp. AZ. *J. Biosci. Bioeng.*, *90*(6), 678–680.

Ogihara, J., Kato, J., Oishi, K., & Fujimoto, Y. **2001**. PP-R, 7-(2-hydroxyethyl)-monascorubramine, a red pigment produced in the mycelia of Penicillium sp. AZ. *J. Biosci. Bioeng.*, *91*(1), 44–47.

Ogihara, J., Kato, J., Oishi, K., Fujimoto, Y., & Eguchi, T. **2000**. Production and structural analysis of PP-V, a homologue of monascorubramine, produced by a new isolate of Penicillium sp. *J. Biosci. Bioeng.*, *90*(5), 549–554.

Ogihara, J., & Oishi, K. **2002**. Effect of Ammonium Nitrate on the Production of PP-V and Effect of ammonium nitrate on the production of PP-V and monascorubrin homologues by Penicillium sp. AZ. *J. Biosci. Bioeng.*, *93*(1), 54–59.

Orozco, S. F. B., & Kilikian, B. V. **2008**. Effect of pH on citrinin and red pigments production by Monascus purpureus CCT3802. *World J. Microbiol. Biotechnol.*, *24*(2), 263–268.

Osmanova, N., Schultze, W., & Ayoub, N. **2010**. Azaphilones: a class of fungal metabolites with diverse biological activities. *Phytochem. Rev.*, *9*(2), 315–342.

Patakova, P. **2013**. Monascus secondary metabolites: production and biological activity. *J. Ind. Microbiol. Biotechnol.*, *40*(2), 169–181.

Pedersen, H., Beyer, M., & Nielsen, J. **2000**. Glucoamylase production in batch, chemostat and fed-batch cultivations by an industrial strain of Aspergillus niger. *Appl. Microbiol. Biotechnol.*, *53*(3), 272–277.

Poulsen, L., Andersen, M. R., Lantz, A. E., & Thykaer, J. **2012**. Identification of a Transcription Factor Controlling pH-Dependent Organic Acid Response in Aspergillus niger. *PLoS One*, *7*(12).

Pusztahelyi, T., Holb, I. J., & Pócsi, I. **2015**. Secondary metabolites in fungus-plant interactions. *Front. Plant Sci.*, *6*(August), 1–23.

Rajendhran, J., Krishnakumar, V., & Gunasekaran, P. **2002**. Optimization of a fermentation medium for the production of Penicillin G acylase from Bacillus sp. *Lett. Appl. Microbiol.*, *35*(6), 523–527.

Rasmussen, K. B. **2015**. *Talaromyces atroroseus. Genome Sequencing, Monascus Pigments and Azaphilone Gene Cluster Evolution*. Technical University of Denmark.

Reichl, U., King, R., & Gilles, E. **1992**. Effect of temperature and medium composition on mycelial growth of Streptomyces tendae in submerged culture. *J Basic Microbiol*, *32*(3), 193–200.

Said, F. M., Brooks, J., & Chisti, Y. **2014**. Optimal C:N ratio for the production of red pigments by Monascus ruber. *World J. Microbiol. Biotechnol.*, *30*(9), 2471–2479.

Santos-Ebinuma, V. C., Roberto, I. C., Francisca, M., Teixeira, S., Jr, A. P., Bioquímico-farmacêutica, D. D. T., Paulo, U. D. S., Biotecnologia, D. De, Lorena, E. D. E. De, & Paulo, U. D. S. **2014**. Improvement of submerged culture conditions to produce colorants by Penicillium purpurogenum. *Brazilian J. Microbiol.*, *742*, 731–742.

Santos-Ebinuma, V. C., Roberto, I. C., Simas Teixeira, M. F., & Pessoa, A. **2013**. Improving of red colorants production by a new Penicillium purpurogenum strain in submerged culture and the effect of different parameters in their stability. *Biotechnol. Prog.*, *29*(3), 778–785.

Santos-Ebinuma, V. C., Teixeira, M. F. S., & Pessoa, A. **2013**. Submerged culture conditions for the production of alternative natural colorants by a new isolated Penicillium purpurogenum DPUA 1275. *J. Microbiol. Biotechnol.*, *23*(6), 802–810.

Sato. **1997**. Identification of major pigments containing D-amino acid units in commercial Monascus pigments. *Chem.Pharm.Bull*, *45*(1), 227–229.

Scervino, J. M., Papinutti, V. L., Godoy, M. S., Rodriguez, M. A., Della Monica, I., Recchi, M., Pettinari, M. J., & Godeas, A. M. **2011**. Medium pH, carbon and nitrogen concentrations modulate the phosphate solubilization efficiency of Penicillium purpurogenum through organic acid production. *J. Appl. Microbiol.*, *110*(5), 1215–1223.

Scotter, M. J. **2011**. Emerging and persistent issues with artificial food colours: natural colour additives as alternatives to synthetic colours in food and drink. *Qual. Assur. Saf. Crop. Foods*, *3*(1), 28–39.

Shao, Y., Lei, M., Mao, Z., Zhou, Y., & Chen, F. **2014**. Insights into Monascus biology at the genetic level. *Appl. Microbiol. Biotechnol.*, *98*(9), 3911–3922.

Shi, K., Chen, G., Pistolozzi, M., Xia, F., & Wu, Z. **2016**. Improved analysis of Monascus pigments based on their pH-sensitive UV-Vis absorption and reactivity properties. *Food Addit. Contam. - Part A*, *33*(9), 1396–1401.

Shi, K., Song, D., Chen, G., Pistolozzi, M., Wu, Z., & Quan, L. **2015**. Controlling composition and color characteristics of Monascus pigments by pH and nitrogen sources in submerged fermentation. *J. Biosci. Bioeng.*, *120*(2), 145–154.

Stadler, M., Anke, H., Dekermendjian, K., Reiss, R., Sterner, O., & Witt, R. **1995**. Novel bioactive azaphilones from fruit bodies and mycelial cultures of the ascomycete Bulgaria inquinans (Fr). *Nat. Prod. Lett.*, *7*(1), 7–14.

Stanbury, P., Whitaker, A., & Hall, S. **2017**. Media for Industrial Fermentations. In *Principles of Fermentation Technology* (3rd ed., pp. 213–272). Elsevier/Pergamon.

Steiner, J., Socha, C., & Eyzaguirre, J. **1994**. Culture conditions for enhanced cellulase production by a native strain of Penicillium purpurogenum. *World J. Microbiol. Biotechnol.*, *10*(562), 280–284.

Sun, X., Yang, X., & Wang, E. **2005**. Chromatographic and electrophoretic procedures for analyzing plant pigments of pharmacologically interests. *Anal. Chim. Acta*, *547*(2), 153–157.

Thrane, U., Rasmussen, K. B., Petersen, B., Rasmussen, S., Sicheritz-Ponten, T., & Mortensen, U. H. **2017**. Genome sequence of Talaromyces atroroseus, which produces red colorants for the food industry. *Genome Announc.*, *5*, e01736-16.

Tolborg, G., Isbrandt, T., Larsen, T. O., & Workman, M. **2017**. Establishing novel cell factories producing natural pigments in Europe. In O. V. Singh (Ed.), *Biopigmentation and Biotechnological Implementation* (First Edit, pp. 23–60). Wiley, NJ, USA.

Torres, F. A. E., Zaccarim, B. R., de Lencastre Novaes, L. C., Jozala, A. F., Santos, C. A. dos, Teixeira, M. F. S., & Santos-Ebinuma, V. C. **2016**. Natural colorants from filamentous fungi. *Appl. Microbiol. Biotechnol.*, *100*(6), 2511–2521.

Torres, P. B., Chow, F., Furlan, C. M., Mandelli, F., Mercadante, A., & dos Santos, D. Y. A. C. **2014**. Standardization of a protocol to extract and analyze chlorophyll a and carotenoids in Gracilaria tenuistipitata Var. Liui. Zhang and Xia (rhodophyta). *Brazilian J. Oceanogr.*, *62*(1), 57–63.

Trinci, A. P. J. **1969**. A Kinetic Study of the Growth of Aspergillus nidulans and Other Fungi. *J. Gen. Microbiol.*, *57*, 11–24.

Tseng, Y. Y., Chen, M. T., & Lin, C. F. **2000**. Growth, pigment production and protease activity of Monascus purpureus as affected by salt, sodium nitrite, polyphosphate and various sugars. *J. Appl. Microbiol.*, *88*(1), 31–37.

Tuli, H. S., Chaudhary, P., Beniwal, V., & Sharma, A. K. **2015**. Microbial pigments as natural color sources: current trends and future perspectives. *J. Food Sci. Technol.*,

52(8), 4669–4678.

Van Hoek, P., Van Dijken, J. P., & Pronk, J. T. **1998**. Effect of Specific Growth Rate on Fermentative Capacity of Baker ' s Yeast. *Appl. Environ. Microbiol.*, *64*(11), 4226–4233.

Vasseur, D. A., Delong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., Mccann, K. S., Savage, V., Tunney, T. D., Connor, M. I. O., Vasseur, D. A., Delong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., Mccann, K. S., Savage, V., Tunney, T. D., & Connor, M. I. O. **2014**. Increased temperature variation poses a greater risk to species than climate warming. *Proc. R. Soc. B*, *281*.

Velmurugan, P., Lee, Y. H., Venil, C. K., Lakshmanaperumalsamy, P., Chae, J.-C., & Oh, B.-T. **2010**. Effect of light on growth, intracellular and extracellular pigment production by five pigment-producing filamentous fungi in synthetic medium. *J. Biosci. Bioeng.*, *109*(4), 346–350.

Vendruscolo, F., Bühler, R. M. M., de Carvalho, J. C., de Oliveira, D., Moritz, D. E., Schmidell, W., & Ninow, J. L. **2015**. Monascus: a Reality on the Production and Application of Microbial Pigments. *Appl. Biochem. Biotechnol.*, 211–223.

Ventura, S. P. M., Santos-Ebinuma, V. C., Pereira, J. F. B., Teixeira, M. F. S., Pessoa, A., & Coutinho, J. A. P. **2013**. Isolation of natural red colorants from fermented broth using ionic liquid-based aqueous two-phase systems. *J. Ind. Microbiol. Biotechnol.*, *40*(5), 507–516.

Vining, L. C. **1990**. Functions of Secondary Structural Complexity of Secondary Metabolites. *Annu. Rev. Microbiol.*, *44*, 395–427.

Wang, Y.-Z., Ju, X.-L., & Zhou, Y.-G. **2005**. The variability of citrinin production in Monascus type cultures. *Food Microbiol.*, *22*(1), 145–148.

Watanabe, T., Mazumder, T. K., Yamamoto, A., Nagai, S., Arimoto-Kobayashi, S., Hayatsu, H., & Terabe, S. **1999**. A simple and rapid method for analyzing the Monascus pigment-mediated degradaton of mutagenic 3-hydroxyamino-1-methyl-5H-pyrido[4,3-b]indole by in-capillary micellar electrokinetic chromatography. *Mutat. Res.*, *444*, 75–83.

Watanabe, T., & Yamamoto, A. **1997**. Separation and determination of Monascus Yellow Pigments for Food by Micellar Electrokinetic Chromatography. *Anal. Sci.*, *13*(August), 571–575.

Wilkins, M. R., & Atiyeh, H. **2011**. Fermentation. In M. T. Dunford (Ed.), *Food and Industrial Bioproducts and Bioprocessing* (1st ed., pp. 185–204). Wiley- Blackwell.

Wissgott, U., & Bortlik, K. **1996**. Prospects for new natural food colorants. *Trends Food Sci. Technol.*, *7*(9), 298–302.

Wong, H., & Koehler, P. **1983**. Production of Red Water-soluble Monascus Pigments. *J. Food Sci.*, *48*, 1200–1203.

Woo, P. C. Y., Lam, C.-W., Tam, E. W. T., Lee, K.-C., Yung, K. K. Y., Leung, C. K. F., Sze, K.-H., Lau, S. K. P., & Yuen, K.-Y. **2014**. The biosynthetic pathway for

a thousand-year-old natural food colorant and citrinin in Penicillium marneffei. *Sci. Rep.*, *4*, 6728.

Woo, P. C. Y., Tam, E. W. T., Chong, K. T. K., Cai, J. J., Tung, E. T. K., Ngan, A. H. Y., Lau, S. K. P., & Yuen, K.-Y. **2010**. High diversity of polyketide synthase genes and the melanin biosynthesis gene cluster in Penicillium marneffei. *FEBS J.*, *277*, 3750–3758.

Workman, M., Andersen, M. R., & Thykaer, J. **2013**. Integrated Approaches for Assessment of Cellular Performance in Industrially Relevant Filamentous Fungi. *Ind. Biotechnol.*, *9*(6), 337–344.

Xiong, X., Zhang, X., Wu, Z., & Wang, Z. **2014**. Accumulation of yellow Monascus pigments by extractive fermentation in nonionic surfactant micelle aqueous solution. *Appl. Microbiol. Biotechnol.*, *99*(3), 1173–1180.

Yang, J., Chen, Q., Wang, W., Hu, J., & Hu, C. **2015**. Effect of oxygen supply on Monascus pigments and citrinin production in submerged fermentation. *J. Biosci. Bioeng.*, *119*(5), 564–569.

Yang, Y., Liu, B., Du, X., Li, P., Liang, B., Cheng, X., Du, L., Huang, D., Wang, L., & Wang, S. **2015**. Complete genome sequence and transcriptomics analyses reveal pigment biosynthesis and regulatory mechanisms in an industrial strain, Monascus purpureus YY-1. *Sci. Rep.*, *5*, 8331.

Yoshimura, M. **1975**. Production of Monascus-Pigment in a Submerged Cultures. *Agr.Biol.Chem*, *39*(9), 1789–1795.

Yu, C. C., Wang, J. J., Lee, C. L., Lee, S. H., & Pan, T. M. **2008**. Safety and mutagenicity evaluation of nanoparticulate red mold rice. *J. Agric. Food Chem.*, *56*(22), 11038–11048.

Yu, J.-H., & Keller, N. **2005**. Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytopathol.*, *43*(114), 437–458.

Zhang, L., Li, Z., Dai, B., Zhang, W., & Yuan, Y. **2013**. Effect of submerged and solid-state fermentation on pigment and citrinin production by Monascus purpureus. *Acta Biol. Hung.*, *64*(3), 385–394.

Zhou, B., Wang, J., Pu, Y., Zhu, M., Liu, S., & Liang, S. **2009**. Optimization of culture medium for yellow pigments production with Monascus anka mutant using response surface methodology. *Eur. Food Res. Technol.*, *228*(6), 895–901.

Appendix A
List of abbreviations

Table A1 Abbreviations used throughout this thesis

$(NH_4)_2SO_4$	Ammonium sulfate
μ_{max}	Maximum specific growth rate
Atr-S	Atrorosin-S
Atr-X	Atrorosin mixture
CS	Carbon source
DAD	Diode array detector
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DW	Dry weight
EFSA	European Food Safety Agency
EtOAc	Ethyl Acetate
FAS	Fatty acid synthase
FDA	Food and Drug Administration
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
IR	infrared
KNO_3	Potassium nitrate
LC	Liquid chromatography
m/z	Mass to charge ratio
MeOD	Deuterated methanol
MeOH	Methanol
MP	*Monascus* pigments
MS	Mass spectrometry
MSG	Monosodium glutamate
NaOH	Sodium hydroxide
NH_4^+	Ammonium
NH_3	Ammonia
NH_4NO_3	Ammonium nitrate
NMR	Nuclear magnetic resonance
NS	Nitrogen source
PKS	Polyketide synthases

POI	Product of interest
PP-O	*P. purpurogenum*- orange
PP-R	*P. purpurogenum*- red,
PP-V	*P. purpurogenum*- violet
PP-Y	*P. purpurogenum*- yellow
rpm	Rotation per minute
SmF	Submerged Fermentation
SN	Supernatant
SPE	Solid phase extraction
spp.	*Species pluralis* (multiple species)
SSF	Solid state fermentation
TFA	Trifluoroacetic acid
UV-Vis	Ultraviolet- visible
vvm	Unit (volume/volume·minute)
Y_{SC}	Yield of CO_2 per substrate
Y_{SP}	Yield of product per substrate
Y_{SX}	Yield of biomass per substrate
Y_{XP}	Yield of product per biomass

Appendix B
List of publications

Peer-Reviewed book chapter:
Tolborg, G., Isbrandt, T., Larsen, T. O., & Workman, M. Establishing novel cell factories producing natural pigments in Europe. *Biopigmentation and Biotechnological Implementation* .(1st Edition, pp. 23-60). Wiley, NJ, USA.

Patent application:
Tolborg, G., Isbrandt, T., Larsen, T. O., & Workman, M. Unique processes yielding a novel class of compounds in *Talaromyces atroroseus.* 2017. P2164PC00.

Submitted and accepted:
Tolborg, G., Isbrandt, T., Larsen, T. O., & Workman, M. Unique processes yielding pure azaphilones in *Talaromyces atroroseus.*
(Accepted by Microbial Cell Factories, 21.03.2018)
Appendix D

To be submitted:
Isbrandt, T., Tolborg, G., Workman, M., & Larsen, T. O. (n.d.). Atrorosins: A new subgroup of *Monascus* pigments from *Taleromyces atroroseus.*
Appendix C

2

ESTABLISHING NOVEL CELL FACTORIES PRODUCING NATURAL PIGMENTS IN EUROPE

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2.1. INTRODUCTION

One of the most distinctive features of manufactured food is its colorful visual perception, and the purchase behavior of modern consumers is driven frequently by appearance. In the supermarket, a particular color can be associated with product freshness and quality. But as consumer awareness increases regarding the link between diet and health, the eventual harmful effects of synthetic colorants are becoming increasingly problematic. The food additive industry thus faces new challenges in providing natural color alternatives and the replacement of chemically synthesized dyes with bio-derived ones.

The market research company MarketsandMarkets estimated the global natural food color market to be worth USD1.1 billion in 2014 and predicted it would reach USD1.7 billion by 2020 (MarketsandMarkets 2014). In 2014, the market for natural colorants drew level with the market share of synthetic colorants, as both represented 34% of the overall food color market (Figure 2.1). The key applications of food colors are confectionary and beverages. Other applications include packaged foods, dairy products, frozen foods, condiments, dressings, functional foods, and pet

Bio-pigmentation and Biotechnological Implementations, First Edition. Edited by Om V. Singh.

Appendix C
Manuscript:
"Atrorosins- a new subgroup of *Monascus* Pigments by *Talaromyces atroroseus*"

Due to conflict with a submitted patent application, this manuscript "Atrorosins- a new subgroup of *Monascus* pigments by *Talaromyces atroroseus*" can first be submitted after the expiration date of the PCT- phase on Nov 8th 2018.

Atrorosins: a new subgroup of *Monascus* Pigments from *Talaromyces atroroseus*

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Abstract

A new series of azaphilone pigments named atrorosins have been isolated from the filamentous fungus *Talaromyces atroroseus*. Atrorosins have a similar azaphilone scaffold as the orange *Monascus* pigment PP-O (*Penicillium purpurogenum*- orange), and are unique by their incorporation of amino acids into the isochromene system. Despite that the atrorosin precursor PP-O, during fermentation was initially produced as two isomers (3:2, *cis:trans* ratio), the atrorosins were surprisingly almost exclusively (99.5%) produced as the *cis* form, indicating that amino acid incorporation into the core polyketide is enzyme catalysed. When grown on KNO_3 as nitrogen source, a whole range of atrorosins is produced, whereas individual atrorosins can be produced selectively during fermentation by supplementing with the desired amino acid.

Keywords: Talaromyces, azaphilone, Monascus pigments, structural elucidation

Introduction

Colouring agents, pigments, or dyes, and their use as food additives have gained increasing attention during the past 10-15 years. Concerns about the safety of synthetic food colorants have made the food industry turn their attention towards natural pigments, such as anthocyanines, carmine and β-carotene. There are however also some challenges regarding these compounds. Anthocyanines and β-carotene have poor long-term and pH stability, which limits their application and the use of carmine has shown to provoke allergic reactions.[1]

Monascus pigments are a class of natural fungal pigments originally identified in filamentous fungi belonging to the genus *Monascus*, however these compounds have also have been found in species belonging to the genera *Penicillium* and *Talaromyces*. Species within the *Monascus* genus have been used as food preservatives and colouring agent for several hundred years and are still used in Asia for production of red rice *koji*[2].

However, *Monascus spp*. have been associated with the production of mycotoxins like citrinin, and other compounds with undesired effects for food ingredients such as lovastatin (monacolin K)[3] and that is why fungi from this genus are not accepted for use on the European and North American market.[4]

Structurally, the *Monascus* pigments represent a subgroup belonging to the class of compounds known as azaphilones, a hugely diverse family of fungal secondary metabolites.[5] Six pigments, first described by Blanc et al[2], are the prototypes for the family of *Monascus* pigments: the yellow monascin and ankaflavin, the orange rubropunctatin and monascorubrin, and the red rubropunctamine and monascorubramine (Figure 1).

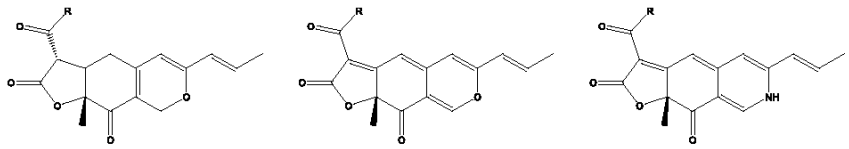


Figure 1. The six original Monascus pigments, with colours spanning from yellow to red.

The filamentous fungus *Talaromyces atroroseus* (previously *Penicillium purpurgenum)*, has recently been shown to secrete large amounts of coloured compounds belonging to the *Monascus* pigments, without production of mycotoxins.[6–10] Some of the pigments are known, such as PP-O, PP-V, PP-Y/monascorubrin, and monascorubramine,[10–13] but a majority of the produced pigments have not been structurally characterised. This study presents the structures of a novel class of *Monascus* pigment, atrorosins, produced by *T. atroroseus*.

Materials and Methods

Strain, Reagents and Instruments

The strain used throughout this study was *Talaromyces atroroseus* IBT 11181 (DTU strain collection). Spores were propagated on CYA agar plates and incubated at 30 °C for 7 days. Spores were harvested with 0.9% sodium chloride solution (NaCl), filtered through mira-cloth, centrifuged and then re-suspended in 0.9% NaCl solution. The spore concentration was determined by using a Burker-Turk counting chamber. All cultivations were inoculated to give an initial spore concentration of 10^6 spores/ml.

All purchased solvents and reagents were acquired from Sigma-Aldrich (St. Louis, Missouri, USA), ultra-pure water was made with a Milli-Q system (Millipore, Burlington, Massachusetts, USA).

Measuring of optical rotation was done on a Perkin-Elmer 341 Polarimeter (Perkin Elmer, Waltham, Massachusetts, USA) using a 10 cm cell.

Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 µm) with a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and remaining for 3 min (0.35 mL/min, 60 °C). An injection volume of 1 µL was used. MS detection was performed in positive detection mode on an Agilent 6545 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 L/min, sheath gas temperature of 300 °C and flow of 12 L/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Mass spectra were recorded at 10, 20 and 40 eV as centroid data for *m/z* 85–1700 in MS mode and *m/z* 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Lock mass solution in 70:30 methanol:water was infused in the second sprayer using an extra LC pump at a flow of 15 µL/min using a 1:100 splitter. The solution contained 1 µM tributylamine (Sigma-Aldrich) and 10 µM Hexakis(2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The $[M + H]^+$ ions (*m/z* 186.2216 and 922.0098 respectively) of both compounds were used.

1D and 2D NMR spectra were recorded on a Bruker Avance 800 MHz spectrometer (Bruker, Billerica, MA, USA) located at the Department of Chemistry at the Technical University of Denmark. NMR spectra were acquired using standard pulse

sequences. The solvent used was either DMSO-d_6 or MeOD, which were also used as references with signals at δ_H = 2.50 ppm and δ_C = 39.5 ppm for DMSO-d_6 and δ_H = 3.31 ppm and δ_C = 49.0 ppm for CD_3OD. Data processing and analysis was done using TopSpin 3.5pl7 (Bruker). *J*-couplings are reported in hertz (Hz) and chemical shifts in ppm (δ). For all natural pigments, 1D proton, edHSQC, and HMBC were recorded, and for atrorosin S, LR-HSQMBC, 1,n-ADEQUATE, and 1,1-ADEQUATE were also measured. Compounds were measured in MeOD, except atrorosin Q which was measured in DMSO-d_6. For the semi-synthesised nature identical pigments, only 1D proton and edHSQC were recorded.

Cultivation

The medium for shake flask cultivations was composed of sucrose (7.5 g/L), glucose (0.375 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H_2O$ (0.1 g/L) and trace metal solution (2 mL/L). The trace metal solution consisted of $CuSO_4 \cdot 5\ H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.04 g/L), $FeSO_4 \cdot 7\ H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2\ H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7\ H_2O$ (8 g/L). The nitrogen source was 0.1 M each of an L-amino acid. Controls were done using 0.1 M of KNO_3. The pH of the medium was adjusted to pH 5 with aqueous NaOH and HCl. Cultivations were carried out in non-baffled shake flasks at 30 °C and 150 rpm in rotary shaking incubators (Forma orbital shaker, Thermo Fisher Scientific, US) with a volume of 100 ml. Samples were taken after 96 hrs. Shake flask experiments were carried out in triplicates. And extractions were done on pooled samples after analysis.

Bioreactor cultivation of PP-O was carried out in duplicates in 1 L bioreactors. The medium for contained sucrose (20 g/L), glucose (1 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H2O$ (0.1 g/L) and trace metal solution (2 mL/L). 2 g/L of KNO_3 were used as nitrogen source. The cultivation was stopped after 53 h at which point it was harvested. The cultivation was performed at 30 °C, 800 RPM, 1 VMM and a pH of 4.5.

The bioreactors were Sartorius 1 L bioreactors (Satorius, Stedim Biotech, Goettingen, Germany) with equivalent working volumes, equipped with 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer's standard procedures. The bioreactor was sparged with sterile atmospheric air and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific, Waltham, MA/USA), calibrated monthly with gas mixtures containing 5 % (v/v) CO_2, 0.04 % (v/v) ethanol and methanol, 1 % (v/v) argon, 5 % (v/v) and 15 % (v/v) oxygen all with nitrogen as carrier gas (Linde Gas, AGA, Enköping, Sweden). The pH was controlled by automatic addition of 2 M NaOH and H_2SO_4.

Purification of PP-O isomers

For purification of PP-O, fermentation liquid was extracted two times with EtOAc to give 1.2 grams of crude extract which was loaded onto an Isolera One (Biotage, Uppsala, Sweden) automated flash system equipped with a diol column and eluted stepwise with dichloromethane (DCM), DCM:EtOAc (1:1), EtOAc, EtOAc:MeOH (1:1), and MeOH. The enriched orange fraction (0.9 g) was subjected to solid phase extraction (SPE) to remove co-eluting impurities. The diol fraction was dissolved in MeOH and loaded onto an equilibrated Oasis WAX solid phase extraction (SPE) column (Waters, Milford, Massachusetts, USA) and washed with 3 column volumes (CVs) of MeOH followed by elution of the orange pigment with 3 CVs of 2% TFA in MeOH. The orange fraction was purified on the same Gilson semi-prep HPLC system as the atrorosins, but using a water/MeOH gradient with 50 ppm TFA and a Kinetex Core-Shell C18 column (250 mm x 10 mm, 5 µm), in order to separate the two isomers.

Purification of atrorosins

Filtered and centrifuged fermentation broth was extracted three times, with 1/3 volume of EtOAc, at pH 3 (adjusted with formic acid). The combined EtOAc phases were evaporated to 100 mL and extracted twice with water (1:1) at pH 8 (adjusted with ammonium hydroxide). The water phase was readjusted to pH 3 with FA and extracted two time with EtOAc, followed by evaporation, to yield >95% pure pigment (a mixture of atrorosins and N-amino acid monascorubramine, ratio>10:1 by LC-DAD-MS). The final purification was performed on a Gilson 332 semi-prep HPLC system equipped with a Gilson 172 diode array detector, using a LUNA II C18 column (250 mm x 10 mm, 5 µm, Phenomenex), with a water/acetonitrile gradient with 50 ppm trifluoroacetic acid (TFA).

Derivatisation of PP-O

For the chemical derivatisation of PP-O to form nature identical atrorosins, PP-O and a 10-15-times molar excess of the amino acid was dissolved in a 1:1 mixture of MeOH and water with 100 ppm TFA (v/v). The solution was heated to 40 °C for 15 min with stirring, and the excess amino acid and unreacted PP-O was removed on an Evolute Express WAX SPE column (Biotage). For lysine, the two isomers were separated by semi-preparative HPLC using a Waters 600 Controller with a 996 photodiode array detector (Waters, Milford, MA, USA) equipped with a Luna II C18 column (250 × 10 mm, 5 µm, Phenomenex), using a H_2O/acetonitrile gradient with 50 ppm TFA.

Results and discussion

Cultivation of *T. atroroseus* leads to isolation of *trans*-PP-O and a novel azaphilone class, atrorosins. Liquid culture fermentation of the filamentous fungus *Talaromyces atroroseus*[6] (IBT11181, DTU strain collection) gave a culture liquid with an intense red colour. Upon analysis of the ethyl acetate extract of the fermentation liquid by Ultra High Performance Liquid Chromatography coupled to Diode Array Detection and High-Resolution tandem Mass Spectrometry (UHPLC-DAD-MS/HRMS), a plethora of different pigmentss were observed. Among them, several known *Monascus* pigments (e.g. PP-O) were identified, along with numerous unknown pigment species.

Careful analysis of the data also showed that PP-O appeared as two distinct isomers in a 3:2 ratio, based on integration of their identical UV spectra. The two isomers were isolated and separated using reverse phase HPLC to generate enough material for structural analysis. From NMR experiments, we were able to identify the two PP-O isomers to be identical, except for having either a *cis*- or a *trans*- form of the double bond between C-2 and C-3. H-2 and H-3 had a coupling constant of J=12.8 Hz, establishing the alkene to be in a Z-configuration.[12,17] NMR spectra for the PP-O isomers can be found in Supporting Information S1, and Supporting Information S2 shows NMR shifts (Table S1) and structures (Figure S1). Since the *trans* form of PP-O has not been reported before, we have named this new compound *trans*-PP-O, and will refer to the known isomer as *cis*-PP-O.

Next we turned our interest towards one of the major unknown red compounds with a mass-to-charge ratio (m/z) of 500.1912, and a UV-VIS absorption spectrum similar to that of known red *Monascus* pigments, i.e. with strong absorption at 430 and 520 nm. Figure 2 shows an example of the extracted UV-VIS profile at 520 nm of a shake flask fermentation using KNO_3 as nitrogen source.

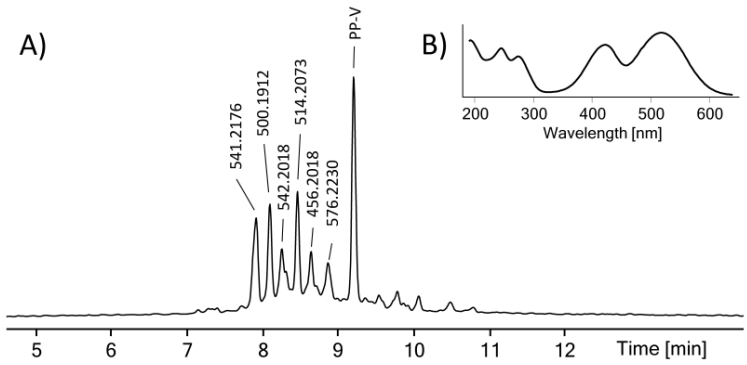


Figure 2. A) UV-VIS chromatogram (520±10nm) of the EtOAc extract from shake flask cultivation of T. atroroseus using KNO3 as nitrogen source. The m/z or identity of the major peaks has been assigned according to the LC-DAD-MS data. B) UV-VIS spectrum of m/z=500.

The unknown red pigment was purified by reverse phase chromatography to give a dark red amorphous solid. From HRMS, a molecular formula of $C_{26}H_{29}N_2O_9$ (DBE=13) was determined (calc. 500.1915, mass accuracy = 0.6 ppm). 1D and 2D

NMR was used to determine the structure of the compound. NMR spectra can be found in Supporting Information S1, and chemical shift values are listed in Table S2. ^{1}H-NMR and HSQC revealed five alkene CH-groups, two methyl groups, seven CH_2-groups, and one alkane CH-group. ^{13}C-NMR and HMBC revealed an additional 11 quaternary carbons of which five were carbonyls, five alkene carbons, and one quaternary alkane. Additionally, two carboxylic acids and an amide were present in the molecule (H and C shifts are listed in table 1).

Tabel B1. Proton and carbon shifts for atrorosin S in CD_3OD.

#	δ_H	δ_C
1	-	166.8
2	6.43 (d, 11.8)	130.9
3	6.94 (d, 11.7)	133.8
4	-	149.7
5	6.81 (s)	119.6
6	-	151.8
7	6.72 (s)	117.7
8	-	168.0
9	-	86.8
9-CH3	1.67 (s)	30.1
10	-	195.4
11	-	98.8
12	8.57 (s)	142.8
13	-	174.7
14	-	125.4
15	-	198.5
16	2.82 (m)	40.9
17	1.59 (quin, 7)	25.8
18	1.31 (m)	23.3/30.0/32.5
19	1.31 (m)	23.3/30.0/32.5
20	1.31 (m)	23.3/30.0/32.5
21	1.31 (m)	23.3/30.0/32.5
22	0.89 (t, 6.2)	13.9
1'	-	169.1
2'	5.12 (s)	67.3
3'	4.28 (dd, 12.2, 5.6) / 4.09 (d, 12.2)	62.5

DQF-COSY showed ^3J-couplings between H-2' and H-3', as well as between H-2 and H-3. H-2 and H-3 had a coupling constant of J=11.8 Hz, suggesting the alkene to be in a Z-configuration. Furthermore, an aliphatic chain consisting of H-16 to H-22 could be identified.

HMBC provided long-range H-C-couplings and could link H-16 and H-17 to the carbonyl C-15. H-7 also showed a coupling to C-15, in addition to couplings to C-6, C-8, C-9, C-11, C13, C14 and C-15, while H-12 had couplings to C-6, C-10 and C-11. H-2 and H-5 both exhibited couplings to C-4. Correlations from H-'2 to C-4 and C-12 was also observed in both HMBC and LR-HSQMBC. Correlations between H-7 and C-8 was determined from 1,1-ADEQUATE.

Based on the structural information obtained from the NMR data, an isoquinoline system could be assembled as the core of the molecule, consisting of C-4 to C-12, with C-13 and C-14 forming a five-membered lactone with C-8 and C-9. Attached to the central ring structure, an α,β-unsaturated carboxylic acid and a methyl group could be linked to C-4 and C9, respectively. A heptanyl chain could be linked to C-14 via the ketone C-15. Finally, a serine moiety was determined to be attached through the isoquinoline nitrogen.

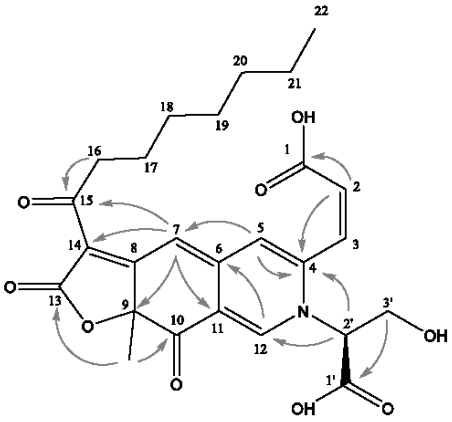


Figure 3. Numbered structure of atrorosin S with key HMBC correlations.

In summary, the compound (Figure 3) is structurally very similar to the *Monascus* pigments PP-O and PP-V[12], but with the incorporation of a serine moiety into the isochromene/isoquinoline system. We have named the compound atrorosin S, using the one-letter amino acid abbreviation to denote which nitrogen-containing compound has been incorporated.

Amino acid feeding expands and focuses the atrorosin catalogue

The discovery of this new azaphilone, stirred our curiosity as to whether *T. atroroseus* was also able to incorporate other amino acids or nitrogen containing compounds into the azaphilone core, as this had previously been described for monascorubrin[14–16]. The LC-MS data from the crude extract was re-examined and several compounds, matching tentative atrorosins were identified. All amino acid derived atrorosins except arginine, histidine, lysine, methionine and proline could be tentatively identified in the extract.

In order to confirm the identities of the pigments, the fungus was cultivated in shake flasks using each of the remaining 19 amino acids as the sole nitrogen source. The ethyl acetate extract of the filtered culture liquid was analysed by UHPLC-DAD-MS/HRMS for each of the 19 cultivations. For proline, no production of the corresponding atrorosins was neither expected nor detected, as the nitrogen atom in the amino acid is a secondary amine. For each of the remaining amino acids, with the exception of tryptophan, the expected atrorosin was to some degree detected in the extract, as illustrated in Figure 4.

Interestingly, upon cultivation with a single amino acid as the sole nitrogen source, the secondary metabolite profile for nearly all experiments changed to contain primarily a single red compound, namely the atrorosin corresponding to the fed amino acid. For a few of the experiments (glycine, cysteine, threonine and tyrosine) other coloured compounds were also detected, as shown in Figure 4, but still far less when compared to shake flask experiments using KNO_3 as nitrogen source (Figure 2).

For glycine and tryptophan, the pigment production was either very low or almost completely absent. In the chromatogram from the glycine extract, some peaks corresponding to unknown red compounds were present, but the one belonging to the tentative atrorosin G was found only in very small amounts, while no red pigments were found in the tryptophan extract. When grown with tyrosine as nitrogen source, in addition to the expected atrorosin Y, significant amounts of both PP-Y and PP-O were also found in the extract.

The lack of pH-control in the shake flasks was most likely the reason why not all of them were equally successful. Growth experiments in bioreactors with well-defined growth conditions has shown that the optimal pH for pigment production was in the quite narrow range of 4-5 (Tolborg *et al.*, in preparation).

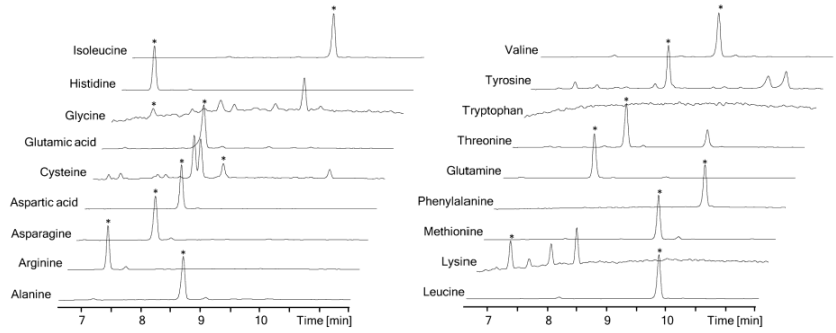


Figure 4. UV-VIS chromatograms (520±10 nm) of EtOAc extracts from shake flask cultivations of T. atroroseus using 18 different amino acids as sole nitrogen source. * indicates the expected atrorosins, confirmed by HRMS.

Because of the poorly controlled conditions in the shake flask cultivations, we were only capable of isolating atrorosins from seven of these (D, E, H, L, M, Q and T) in addition to the original atrorosin S. Rather than carrying out bioreactor

cultivations with each of the remaining amino acids, a single four litre fermentation, was done and harvested at the time where the PP-O concentration was at its highest, with the aim of derivatising PP-O to produce all nature identical atrorosins.

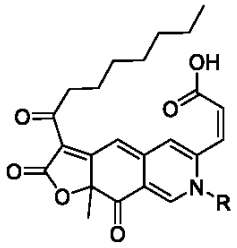
Semi-synthesis of nature identical atrorosins from *cis*-PP-O

During the submerged shake flask cultivation, L-amino acids were added in order to produce single atrorosins, and it was therefore expected that these would also contain the L-amino acid upon excretion. However, to ensure that no isomerisation had taken place, we synthesised nature identical atrorosins from *cis*-PP-O and both L- and D-amino acids to compare these against natural pigments isolated from the fermentation broth.

The chemical incorporation of amine containing compounds into the backbone of the *Monascus* pigment monascorubrin/PP-Y has previously been reported[15] and we have shown that the same is possible with PP-O under basic conditions, however with poor selectivity as several isomers were formed (Tolborg *et al., in preparation*).

In the present study, we learned that though slower, the acid catalysed reaction provides better stereoselectivity during the *in vitro* incorporation of e.g. amino acids, compared to the base catalysed reaction (data not shown). By reacting *cis*-PP-O with amino acids in a 1:1 mixture of water and methanol acidified with 100 ppm triflouroacetic acid we could almost quantitatively obtain each of the desired atrorosins (HPLC data in Supporting Information S4, Figure S2). ^{1}H-NMR and HSQC were recorded for each of the synthesised compounds, and could together with accurate mass and fragmentation patterns from HR-MS/MS confirm formation of the expected products (NMR spectra and chemical shifts for each of the semi-synthesised atrorsins can be found in the Supporting Information S1 and S3). For atrorosin E and S we also incorporated the D-amino acid into *cis*-PP-O to compare the optical rotation with the natural compound in order to confirm whether the amino acid is modified before being excreted by the fungus (values listed in Supporting Information S5). These experiments confirmed our hypothesis, that the amino acids in the natural atrorosins are indeed the more naturally abundant L-amino acids and that the natural atrorosins all contain L-amino acids, when these are added during cultivation.

The remaining atrorosins not isolated from the shake flask cultivations were also made from *cis*-PP-O, and ^{1}H-NMR and HSQC spectra were obtained for these. NMR spectra, and proton and carbon shifts for all characterised atrorosins as well as both isomers of PP-V can be found in Supporting Information S1 and S3, and their structures (except atrorosin S) are shown in Figure 5.



R= Ala: Atrorosin A | His: Atrorosin H | Gln: Atrorosin Q
Cys: Atrorosin C | Iso: Atrorosin I | Arg: Atrorosin R
Asp: Atrorosin D | Lys: Atrorosin K | Thr: Atrorosin T
Glu: Atrorosin E | Leu: Atrorosin L | Val: Atrorosin V
Phe: Atrorosin F | Met: Atrorosin M | Trp: Atrorosin W
Gly: Atrorosin G | Asn: Atrorosin N | Tyr: Atrorosin Y

Figure 5. Chemical structure of 18 atrorosins.

In summary, the filamentous fungus *T. atroroseus* has been found to excrete high amount of a new class of red azaphilone pigments, atrorosins. These are all derivatives of the orange pigment *cis*-PP-O, differing only by their incorporation of L-amino acids, which can either be naturally incorporated during standard growth conditions to give a range of different compounds, or be selectively incorporated by feeding single amino acids during cultivation to give only one version of the pigment. On the contrary to PP-O, that was showed to be naturally produced in both a *cis*- and *trans* form, we find it highly surprising that the atrorosins were almost exclusively (>99.5%) produced as the *cis* isomer. We therefore propose that amino acid incorporation into the core polyketide skeleton in *T. atroroseus* might be enzyme assisted, something that to the best of our knowledge has not previously been suggested, and could even involve transformation of the C-2/C-3 alkene in *trans*-PP-O into the *cis* form seen in atrorosins, since neither *trans*-PP-O nor "*trans*-atrorosins" could be detected after incorporation of amino acids.

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The authors would like to acknowledge Kasper Enemark-Rasmussen at the Department of Chemistry, Technical University of Denmark, for running NMR experiments.

References

(1) Rohrig, B. *ChemMatters* **2015**, No. October/November, 5.
(2) Blanc, P. J.; Loret, M. O.; Santerre, a L.; Pareilleux, A.; Prome, D.; Prome, J. C.; Laussac, J. P.; Goma, G. *J. Food Sci.* **1994**, *59* (4), 862.
(3) Yang, J.; Chen, Q.; Wang, W.; Hu, J.; Hu, C. *J. Biosci. Bioeng.* **2015**, *119* (5), 564.
(4) Eisenbrand, G. *Mol. Nutr. Food Res.* **2006**, *50* (3), 322.
(5) Gao, J.; Yang, S.; Qin, J. *Chem. Rev.* **2013**, *113* (7), 4755.

(6) Frisvad, J. C.; Yilmaz, N.; Thrane, U.; Rasmussen, K. B.; Houbraken, J.; Samson, R. *PLoS One* **2013**, *8* (12), e84102.
(7) Mapari, S. Prodcution of Monascus-like azaphilone pigments. EP 2262862 B1, 2007.
(8) Mapari, S. a S.; Nielsen, K. F.; Larsen, T. O.; Frisvad, J. C.; Meyer, A. S.; Thrane, U. *Curr. Opin. Biotechnol.* **2005**, *16* (2), 231.
(9) Mapari, S. a S.; Meyer, A. S.; Thrane, U. *J. Agric. Food Chem.* **2006**, *54* (19), 7027.
(10) Ogihara, J.; Kato, J.; Oishi, K.; Fujimoto, Y.; Eguchi, T. *J. Biosci. Bioeng.* **2000**, *90* (5), 549.
(11) Ogihara, J.; Kato, J.; Oishi, K.; Fujimoto, Y. *J. Biosci. Bioeng.* **2001**, *91* (1), 44.
(12) Arai, T.; Kojima, R.; Motegi, Y.; Kato, J.; Kasumi, T.; Ogihara, J. *Fungal Biol.* **2015**, *119* (12), 1226.
(13) Ogihara, J. **2001**, *93* (1), 54.
(14) Hajjaj, H.; Klaebe, A.; Loret, M. O.; Tzedakis, T.; Goma, G.; Blanc, P. J. *Appl. Environ. Microbiol.* **1997**, *63* (7), 2671.
(15) Lin, T. F.; Yakushijin, K.; Bt, G. H.; Demain, A. L.; Buchi, G. H.; Demain, A. L.; Bt, G. H.; Demain, A. L. *J. Ind. Microbiol.* **1992**, *9* (3–4), 173.
(16) Jung, H.; Kim, C.; Kim, K.; Shin, C. S. *J. Agric. Food Chem.* **2003**, *51* (5), 1302.
(17) Ogihara, J.; Oishi, K. *J. Biosci. Bioeng.* **2002**, *93* (1), 54.

Appendix D
Manuscript: "Unique fermentation processes yielding pure azaphilones in *Talaromyces atroroseus*"

The manuscript "Unique fermentation processes yielding pure azaphilones in *Talaromyces atroroseus"* was accepted in April 2018 by Microbial Cell Factories. Subsequently, submission was withdrawn to not be in conflict with a submitted patent application. It will be re- submitted after the expiration date of the PCT-phase on Nov 8th 2018.

Unique processes yielding pure azaphilones in *Talaromyces atroroseus*

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Abstract

Azaphilones are a class of fungal pigments, reported mostly in association with Monascus species. In Asian countries they are used as food colorants under the name of "red rice" and their production process is well described. One major limitation of current production techniques of azaphilones is that they always occur in a mixture of yellow, orange and red pigments. These mixtures are difficult to control and to quantify. This study has established a controlled and reproducible cultivation protocol to selectively tailor production of individual pigments during a submerged fermentation using another fungal species capable of producing azaphilone pigments, Talaromyces atroroseus, using single amino acids as the sole nitrogen source. The produced azaphilone pigments are called atrorosins and are amino acid derivatives of the known azaphilone pigment Penicilium purpurogenum- orange (PP-O), with the amino acid used as nitrogen source incorporated into the core skeleton of the azaphilone. This strategy was successfully demonstrated using 18 natural amino acids and the non-proteinogenic amino acid ornithine. Two cultivation methods for production of the pure serine derivative (atrorosin-S) have been further developed, with yields of 0.9 g/L being obtained. In vitro synthesis of atrorosins was demonstrated by incorporation of an amino acid precursor into PP-O. Chemical in vitro synthesis suggested the importance of the pH for amino acid incorporation. Yielding pure atrorosins through switching from potassium nitrate to single amino acids as nitrogen source allows for considerably easier downstream processing and thus further enhances the commercial relevance of azaphilone producing fungal cell factories.

Keywords: filamentous fungi, azaphilone pigments, natural colorants, amino acid derivative, submerged cultivation, *in vitro* synthesis

Background

Natural food colorants are at the focal point of interest due to the growing consumer awareness of possible harmful effects of synthetic colorants (Amchova, Kotolova, & Ruda-Kucerova, 2015; Scotter, 2011). With the increasing importance of diet and health, the food additive industry faces new challenges in providing natural colour alternatives. Currently, most industrially applied natural colorants are extracted directly from natural sources *e.g.* betanins (beet root *Beta vulgaris*), carminic acid (extracted from the female insect *Dactylopius coccus* (Dapson, 2007)) or lycopene (tomato *Solanum lycopersicum*). Their production is highly dependent on the supply of raw ingredients and are subject to seasonal variation both in regards to quantity and quality (Wissgott & Bortlik, 1996). These limitations can potentially be overcome by exploring new sources for natural pigments such as microorganisms (L Dufossé, 2006). Fungi have an excellent potential in this regard, as they are known to naturally biosynthesize and excrete diverse classes of secondary metabolites including pigments within a broad range of colours (Laurent Dufossé, Fouillaud, Caro, Mapari, & Sutthiwong, 2014).

One well- studied pigment producing fungal genus is Monascus, which has been used for many years in the manufacturing of traditional foods in Asian countries (Arunachalam & Narmadhapriya, 2011). Pigments from Monascus are referred to as "Monascus pigments" and describe a mixture of chemical compounds called azaphilones including yellow, orange, and red constituents. Monascus pigments have been associated with production of the mycotoxin citrinin, excluding their use as food colorants in western countries (Liu, Wu, Su, Ping Chung, & Yu, 2005). Monascus pigment biosynthesis is highly dependent on the medium conditions, and different colour shades can be produced through nitrogen source selection (Shi et al., 2015). The pyranoquinone bicyclic core of the azaphilones is often highly oxygenated and can react with amines by exchanging the pyran oxygen with nitrogen (Stadler et al., 1995). This exchange leads to a colour shift from yellow/orange to red. It has been demonstrated in *Monascus* that supplementing ammonium nitrate with a specific amino acid leads to the incorporation of the amino acid into the pigment core structure resulting in the formation of new pigment derivatives in the pigment cocktail (Jung, Kim, Kim, & Shin, 2003).

Large scale *Monascus* pigment production, therefore results in a mixture of different *Monascus* pigments (Woo et al., 2014). The disadvantage is that the exact pigment composition is difficult to control and has high batch to batch variation. For standardised and controlled production, needed to obtain regulatory approval for food applications, it would be preferable to obtain a single pigment.

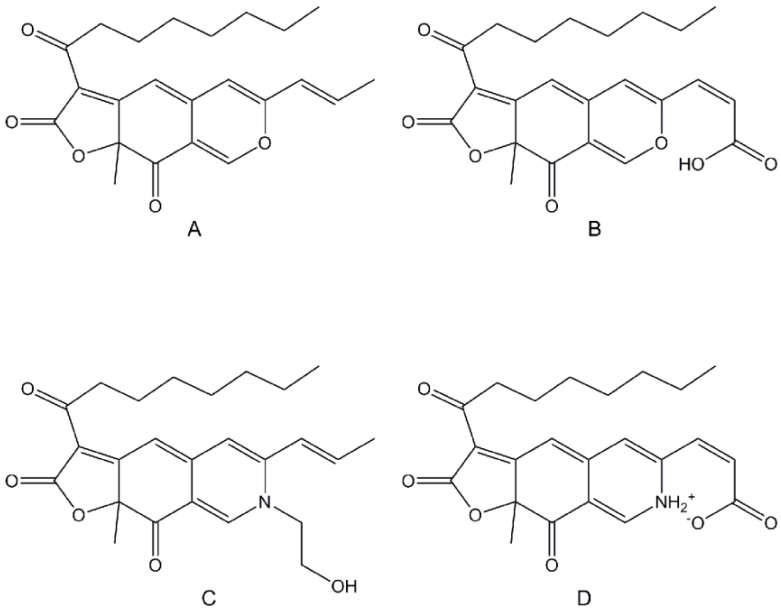


Figure C1 Chemical structures of *Monascus* pigments which have been identified in *T. atroroseus*: Penicillium Purpurogenum –Yellow (PP-Y)(Jun Ogihara & Oishi, 2002), Penicillium Purpurogenum –Orange (PP-O) (Jun Ogihara & Oishi, 2002), Penicillium Purpurogenum –Red (PP-R) (Jun Ogihara, Kato, Oishi, & Fujimoto, 2001), Penicillium Purpurogenum –Violet (PP-V) (Jun Ogihara & Fujimotoz, 2000),

Monascus pigments were first described in association with *Monascus* species, but have henceforth been identified in numerous other species (Gao, Yang, & Qin, 2013). One promising *Monascus*-like pigment producer was identified by *Mapari et al.* under the name *Penicillium purpurogenum* and was later reclassified by *Frisvad et al.* as a *Talaromyces atroroseus* IBT 11181 (Frisvad et al., 2013; Mapari, Meyer, Thrane, & Frisvad, 2009). When cultivated on plates, *T. atroroseus* excreted a bright red colour and in submerged cultivation *T. atroroseus* demonstrated promising pigment profile and, most importantly absence of mycotoxins (Mapari et al., 2009). *T. atroroseus* has been studied previously (Arai et al., 2012, 2013, 2015; Kojima et al., 2016; Mapari et al., 2009; J Ogihara, Kato, Oishi, Fujimoto, & Eguchi, 2000; Jun Ogihara & Fujimotoz, 2000; Jun Ogihara et al., 2001; Jun Ogihara & Oishi, 2002) and so far six *Monascus*- like pigments have been structurally presented (Frisvad et al., 2013; Mapari et al., 2009; J Ogihara et al., 2000; Jun Ogihara & Fujimotoz, 2000; Jun Ogihara et al., 2001). Four of these pigments are illustrated in Figure C1. A novel class of pigments has recently been identified in *T. atroroseus*. They are referred to as atrorosins and are derivatives of the

known pigment *Penicilium purpurogenum*- violet (PP-V) (*Isbrandt et al.* in progress).

To tailor pigment production in submerged cultivations, both the physiological conditions for optimal fungal growth as well as the conditions for product bio-synthesis need to be considered (Papagianni, 2004). Media constituents including carbon and nitrogen sources as well as process parameters such as pH have shown to significantly affect pigment production in *Monascus* (Broder & Koehler, 1980; Lin & Demain, 1991; Said, Brooks, & Chisti, 2014; Yoshimura, 1975). However, optimal conditions for pigment production are not always in accordance with optimal conditions for growth.

As with *Monascus* species, the pigment profile from *T. atroroseus* is strongly influenced by the choice of nitrogen source (Santos-Ebinuma, Roberto, Simas Teixeira, & Pessoa, 2013). In *T. atroroseus*, ammonium nitrate together with yeast extract promotes production of violet/red pigments *Penicillium purpurogenum*- violet (PP-V) and *Penicilium purpurogenum*- red (PP-R)), but when yeast extract is used as sole nitrogen source PP-V and PP-R production is replaced by production of *Penicillium purpurogenum- orange* (PP-O) and *Penicilium purpurogenum*- yellow (PP-Y) giving a yellow/orange colour profile (Jun Ogihara & Oishi, 2002).

The aim of this study was to investigate the effect of using amino acids as the sole nitrogen source on the pigment biosynthesis in the promising T. atroroseus IBT 11181. Amino acids were screened in submerged shake flask cultivations as the sole nitrogen source to assess the differences in biomass accumulation and pigment production. Based on these results, two cultivation methods to produce single atrorosins, instead of a cocktail of pigments as seen in Monascus, has been developed. Finally, to further investigate the atrorosin biosynthesis and mechanism of amino acid incorporation, in vitro synthesis of atrorosins was demonstrated using PP-O and an amino acid as precursors, yielding pure atrorosins.

Methods

Reagents

All reactants and media ingredients were purchased from Sigma-Aldrich GmbH, Steinheim, Germany.

Strain and propagation

The strain used in this study was *Talaromyces atroroseus* IBT 11181 (DTU strain collection). *T. atroroseus* spores were propagated on CYA agar plates and incubated at 30 °C for 7 days. Spores were harvested with 0.9% sodium chloride

solution (NaCl), filtered through mira-cloth, centrifuged and then re-suspended in 0.9% NaCl solution. The spore concentration was determined by using a Burker-Turk counting chamber. All cultivations were inoculated to give an initial spore concentration of 10^6 spores/ ml.

Submerged Cultivation

The medium for cultivation in shake flasks was composed of sucrose (7.5 g/L), glucose (0.375 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H_2O$ (0.1 g/L) and trace metal solution (2 mL/L). The trace metal solution consisted of $CuSO_4 \cdot 5\ H_2O$ (0.4 g/L), $Na_2B_4O_7 \cdot 10\ H_2O$ (0.04 g/L), $FeSO_4 \cdot 7\ H_2O$ (0.8 g/L), $MnSO_4 \cdot H_2O$ (0.8 g/L), $Na_2MoO_4 \cdot 2\ H_2O$ (0.8 g/L), $ZnSO_4 \cdot 7\ H_2O$ (8 g/L). The nitrogen source was 0.1 M each of L-amino acid. 0.1 M of KNO_3 was used as the control. The pH of the medium was adjusted to pH 5 with aqueous NaOH and HCl. Cultivations were carried out in non-baffled shake flasks at 30 ºC and 150 rpm in rotary shaking incubators (Forma orbital shaker, Thermo Fisher Scientific, US) with a volume of 100 ml. Samples were taken after 96 hrs. Shake flask experiments were carried out in triplicates.

Submerged cultivation with serine as the sole nitrogen source was carried out in a 1 L bioreactor. For cultivation in bioreactors, 20 g/L sucrose was used, but the other constituents were kept at the concentrations used in shake flasks. The cultivation was run at 30 °C, 800 RPM, 1 VVM and a pH of 4.5. The bioreactor experiments were carried out in duplicates.

Two-step cultivations were carried out in 1 L bioreactors. The medium for two-step cultivation contained sucrose (20 g/L), glucose (1 g/L), KH_2PO_4 (10 g/L), NaCl (1 g/L), $MgSO_4 \cdot 7\ H_2O$ (2 g/L), KCl (0.5 g/L), $CaCl_2 \cdot H2O$ (0.1 g/L) and trace metal solution (2 mL/L). For the first step, 2 g/L of KNO_3 were used as nitrogen source. After 53 h of cultivation the concentration of PP-O in the medium was measured and a 10-fold concentration of serine was added (1 g/L) to induce formation of the amino acid derivative and to ensure optimal conversion. The cultivation was performed at 30 °C, 800 RPM, 1 VMM and a pH of 4.5.

All reactor based cultivations were carried out in duplicates using Sartorius 1 L bioreactors (Satorius, Stedim Biotech, Goettingen, Germany) with equivalent working volumes and equipped with 2 Rushton six-blade disc turbines. The pH electrode (Mettler Toledo, OH/USA) was calibrated according to manufacturer's standard procedures. The bioreactor was sparged with sterile atmospheric air and off-gas concentrations of oxygen and carbon dioxide were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fischer Scientific, Waltham, MA/USA), calibrated monthly with gas mixtures containing 5 % (v/v) CO_2, 0.04 % (v/v) ethanol and methanol, 1 % (v/v) argon, 5 % (v/v) and 15 % (v/v) oxygen all with nitrogen as carrier gas (Linde Gas, AGA, Enköping, Sweden). The pH was controlled by automatic addition of 2 M NaOH and H_2SO_4.

Sampling

Samples for dry weight (DW) analysis, HPLC, absorbance analysis and LC-MS analysis were taken regularly throughout the cultivations. Samples intended for HPLC, absorbance and LC-MS were filtered through a sterile Statorius Stedim filter (BRAND) with a pore size of 0.45 µm in order to separate biomass from the filtrate.

Dry Weight analysis

DW was assessed on filters which were pre-dried in a microwave for 20 min, kept in a desiccator for a minimum of 10 min and weighed. For DW analysis, the filters were placed in a vacuum filtration pump and ca. 10 ml of culture broth was added. Subsequently the filters with the biomass were dried in a microwave for 20 min and kept in a desiccator for a minimum of 10 min before being re-weighed. The weight of the biomass was determined as the difference of the filter weight before and after sample application.

Analysis of extracellular metabolites by HPLC

Culture samples were filtered through a 0.45 µm cellulose acetate filter (Frisenette, Knebel, Denmark). The samples were frozen and kept at -20ºC until analysis. Glucose, glycerol, pyruvate, succinate, acetate and ethanol were detected and quantified using an Agilent 1100 HPLC system equipped with a refractive index and Diode array detector (Agilent Technologies, Waldbronn, Germany) and with an Aminex HPX-87H cation-exchange column (BioRad, Hercules, Ca, USA). Compounds were separated by isocratic elution at 30ºC, with 5 mM H2SO4 at a flow rate of 0.8 mL min-1. Quantification was performed using a six-level external calibration curve with glucose and pyruvate detected at a wavelength of 210 nm and sucrose, fructose, succinate, glycerol, acetate and ethanol by refractive index measurements.

Extraction and purification of Pigments

Filtered and centrifuged fermentation broth was extracted three times, with 1/3 volume of EtOAc, at pH 3 adjusted with formic acid (FA). The combined EtOAc phases were evaporated to 100 mL and extracted twice with milli-Q water (1:1) at pH 8 adjusted with ammonium hydroxide (NaOH). The water phase was readjusted to pH 3 with FA and extracted two times with EtOAc, followed by evaporation, to yield >95% pure pigment (a mixture of atrorosins and N-amino acid monascorubramine, ratio>10:1). The two pigments were separated on a Gilson 332 semi-prep HPLC system equipped with a Gilson 172 diode array detector, using a LUNA II C18 column (250 mm x 10 mm, 5 µm, Phenomenex), with a water/acetonitrile gradient.

LC-MS analyses

Ultra-high Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 250 mm, 2.7 µm) with a linear gradient consisting of water and acetonitrile both buffered with 20 mM FA, starting at 10% B and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and remaining for 3 min (0.35 mL/min, 60 °C). An injection volume of 1 µL was used. MS detection was performed in positive detection mode on an Agilent 6545 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, gas flow of 8 L/min, sheath gas temperature of 300 °C and flow of 12 L/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Mass spectra were recorded at 10, 20 and 40 eV as centroid data for *m/z* 85–1700 in MS mode and *m/z* 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Lock mass solution in 70:30 methanol:water was infused in the second sprayer using an extra LC pump at a flow of 15 µL/min using a 1:100 splitter. The solution contained 1 µM tributylamine (Sigma-Aldrich) and 10 µM Hexakis (2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK) as lock masses. The $[M + H]^+$ ions (*m/z* 186.2216 and 922.0098 respectively) of both compounds was used.

Quantitative Analysis of the Pigment

The absorbance values of the individual pigment solutions were determined using a Synergy 2 photo spectrum (BioTek, Germany) and a 96 well microtiter plate. 150 µL of sample broth of each amino-acid-pigment-solution were scanned in the range of 200-700 nm and maximum absorbance values were determined. Absorbance at 500 nm indicated presence of red pigments. A standard curve of an orange and red pigment was used to calculate the concentration in the medium. For the amino acids, where no standard curve was available the absorbance is given in AU/150µL.

Chemical derivatization of PP-O to form atrorosins

300 µL of a 0.05 M solution of different amino-group containing reactants were added to 300 µL of a 0.003 M solution of PP-O. The tested reactants were L-glutamine (pH 2 and pH 9), L- glutamic Acid (pH 2 and pH 9), L- ornithine (pH 2 and pH 9), L- tryptophan (pH 2 and pH 9), L- serine (pH 2), and D- serine (pH 2). The pH was adjusted to pH 9 with formic acid. The samples were vortexed and analysed by LC-MS.

Results

Influence of amino acid supplements on biomass accumulation and pigment production

To assess the potential of the canonical amino acids as sole nitrogen source for biomass and pigment production, all 20 natural amino acids and ornithine were tested in shake flask experiments in duplicates. A control containing KNO_3 as nitrogen source was also cultivated to benchmark biomass and pigment production. Accumulated biomass values and the absorbance intensities for all tested 21 amino acids and the control are shown in Figure C2.

Biomass accumulation was highest with glutamine (6.55 ± 0.3 g/L), followed by proline (6.05± 0.3 g/L), alanine (5.48 ± 0.01 g/L) and ornithine (4.83 ± 0.07 g/L). The use of arginine (4.45 ± 0.02 g/L), asparagine (4.4 ± 0.03 g/L), aspartic acid (4.26 ± 0.5 g/L) and glutamic acid (4.49 +0.24 g/L) gave similar results. The control with KNO_3 as nitrogen source yielded only 2.77 +0.06 g/L biomass. Histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan and tyrosine had low biomass accumulation below 2 g/L.

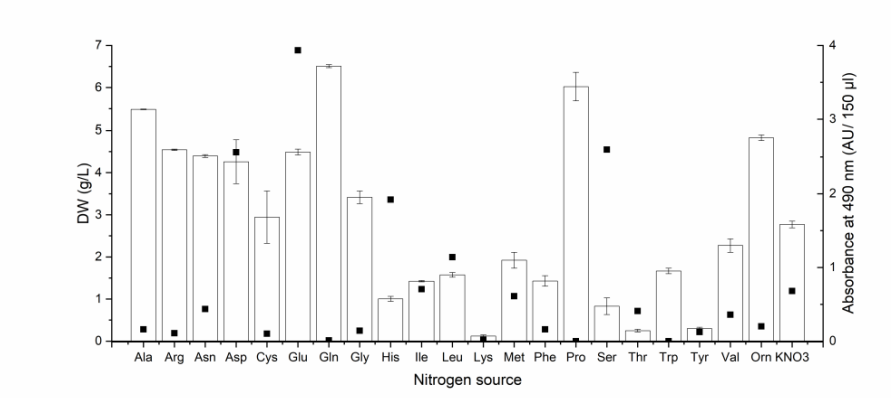


Figure C2 Biomass values (bar diagram) and absorbance intensity of fermentation broth (•) of *T. atroroseus* cultures in shake flasks with single amino acids as sole nitrogen source after 96 h. Cultivation in shake flasks were performed at 30 °C, 150 rpm and at a starting pH of 5.

Regarding pigment production, amino acids which yielded high biomass concentrations did not necessarily produce high amounts of pigment. There was no pigment production with proline and tryptophan, and very low

absorbance intensities were measured when adding alanine and ornithine. Other low absorbance intensity (below 1) yielding amino acids were arginine, asparagine, cysteine, glutamic acid, glycine, isoleucine, lysine, methionine, phenylalanine, tyrosine and valine. The control with potassium nitrate had an absorbance intensity of 0.68 AU/150µl. The highest absorbance intensity was obtained with glutamic acid (3.9 AU/150µl), followed by aspartic acid, serine and histidine. In contrast to glutamic acid, glutamine did not lead to high pigment concentration.

Data for absorbance intensity/ biomass are shown in Table C1 for the four amino acids with the most favourable ratios (histidine, serine, threonine, glutamic acid and the control KNO_3; other data not shown).

Table C 1 Absorbance intensity/ biomass, name and [M/Z] of the corresponding atrorosin of the 4 highest yielding nitrogen sources and the control

Nitrogen source	Yield Y_{XP} (Intensity (AU/150µl) / biomass g/L)	Name of atrorosin(s) detected	[m/z] (Da)
His	1.91±0.06	Atrorosin-H	m/z= 550.2184
Ser	3.13±0.02	Atrorosin-S	m/z= 500.1915
Thr	1.63±0.03	Atrorosin-T	m/z= 514.2072
Glu	0.88±0.07	Atrorosin-E	m/z= 542.2021
KNO_3	0.25±0.08	PP-V	m/z= 412.1755
		Atrorosin-T	m/z= 514.2072
		Atrorosin-Q	m/z= 541.2181
		Atrorosin-S	m/z= 500.1915
		Atrorosin-E	m/z= 542.2021

LC-MS analysis of fermentation broth from shake flasks

When *T. atroroseus* was cultivated with potassium nitrate as nitrogen source, a plethora of different azaphilone pigments was produced (Figure C3A). This cocktail of pigments contained not only the novel group of pigments called atrorosins but also known monascorubramine derivatives. The most abundant pigments from *T. atroroseus* on potassium nitrate were PP-V and atrorosin derivatives from threonine, glutamine, serine and glutamate. Apart from glutamine, these amino acids were the same high yielding amino acids from the one step cultivation presented in table 1. Atrorosin- H (histidine derivative) was, however, not detected in the pigment mixture. On the contrary, when a single amino acid was used as the sole nitrogen source, only one single type of azaphilone pigment was produced, namely the respective atrorosin with the fed amino acid. Extracted UV-VIS- chromatograms at 520 nm from the

cultivations on histidine, serine, threonine, glutamate and KNO_3 are shown in Figure C3A).

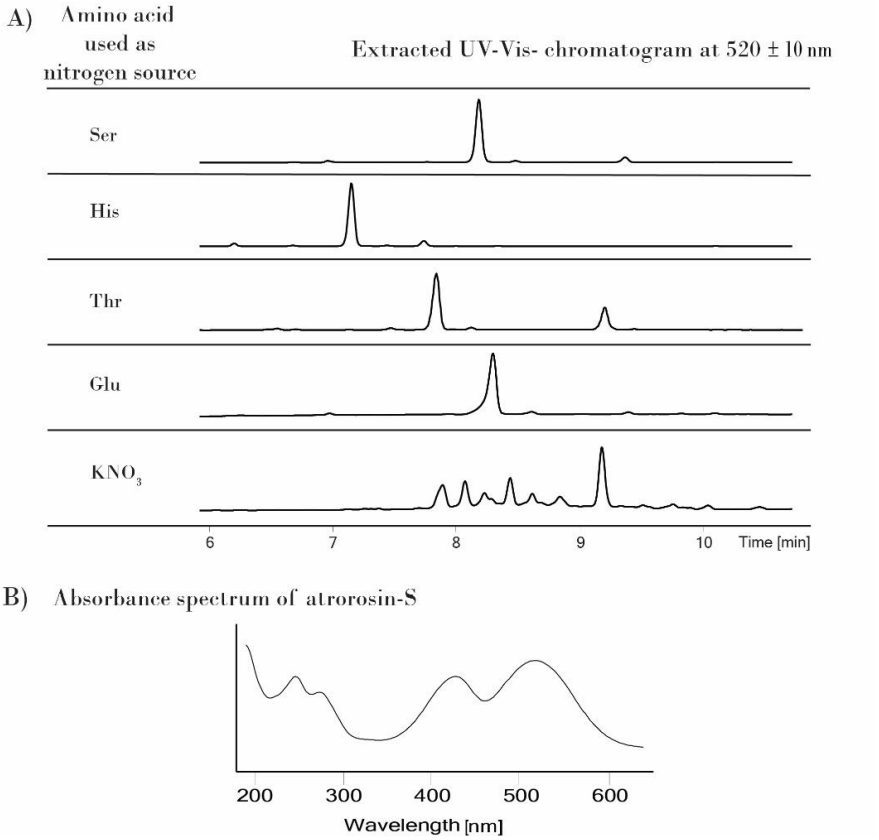


Figure C3 A) UV-Vis-chromatograms (taken at 520±10 nm) of fermentation broth from cultivation with KNO3 and different amino acids as nitrogen source. Shake flasks cultivation at 30 °C and 150 rpm. B) Exemplary absorbance spectrum of atrorosin-S from the same cultivation

In these chromatograms compounds that absorb light at 520 nm and therefore appear red were detected. As only one or two peaks were present in the chromatograms for the single amino acid cultivation, the purity of the fermentation broth with regard to azaphilone was greatly increased compared to the KNO_3 control. The highest peak corresponded to the produced atrorosin. The smaller peak in the cultivation with histidine, serine and threonine corresponded to an amino-acid derivative without the carboxylic acid. All atrorosins, regardless of their amino acid incorporation, had absorbance

maxima at 245 nm, 275nm, 420 nm and 520 nm. As an example, the absorbance spectrum from atrorosin-S is shown Figure C3B).

Lab-scale amino acid-derivative production in the bioreactor

The superior pigment purity with serine as the sole nitrogen source was the basis for bioreactor cultivations. Cultivation in bioreactors allows for pH control, controlled aeration and sparging which should lead to both higher biomass and pigment production. Two cultivation methods were developed, one using only serine as the nitrogen source similar to the shake flask and secondly a two-step process with an initial concentration of potassium nitrate to allow for biomass accumulation before a nitrogen switch to serine. Previous experiments indicated that under nitrogen limiting conditions, synthesis of PP-O was favoured and no nitrogen was incorporated in the pigment core structure. This knowledge was applied in the two-step cultivation. First, PP-O production was promoted in the first step by inducing nitrogen-limited conditions and then, in a second step, with the addition of serine, PP-O was converted into atrorosin-S. Cultivation with KNO_3 as nitrogen source served as a control.

Figure C4 shows the cultivation and pigment profiles. The cultivation shown in Figure C4A) had 0.1 M KNO_3 as nitrogen source, the cultivation profile as shown in Figure C4B) used 0.1 M serine as sole nitrogen source and cultivation shown in Figure C4C) started with an initial concentration of 0.02 M KNO_3 and after depletion approx. 55 hours, 0.01 M of sterile serine was added to the bioreactor.

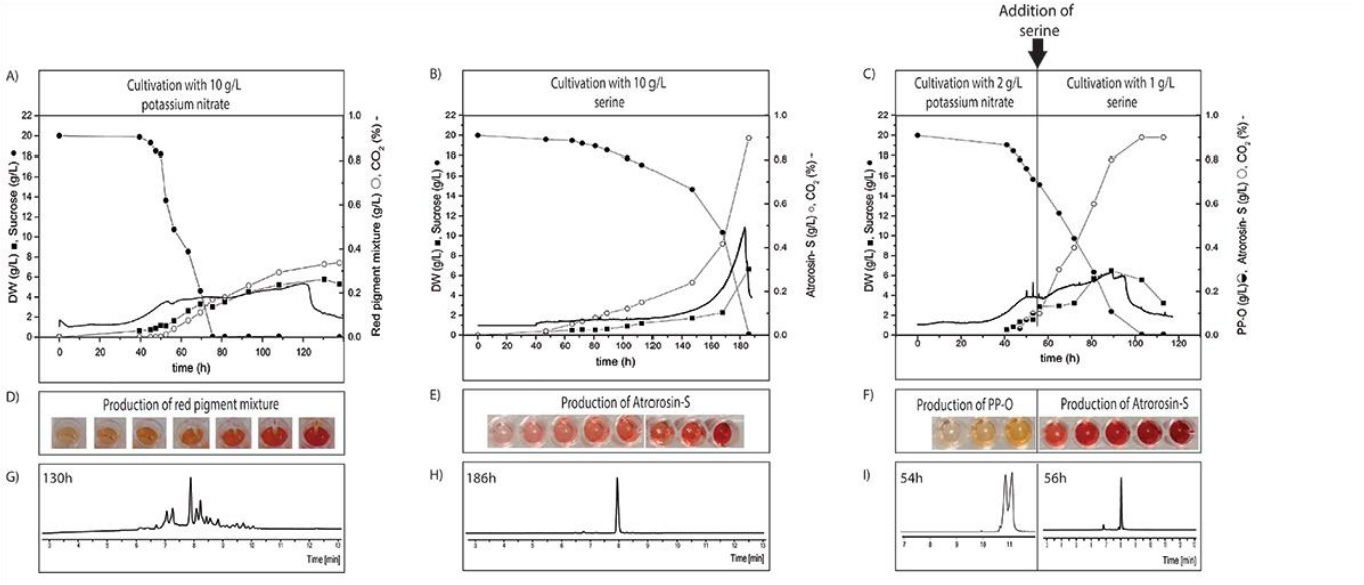


Figure C 4 Time course of bioreactor cultivation with A) KNO_3, B) serine and C) with nitrogen source shift (two-step cultivation). Color profile of fermentation supernatant from cultivation with D) KNO_3, E) serine and F) of the two-step cultivation. UV-Vis chromatogram (520±10 nm) taken of the fermentation supernatant at the end of the cultivation from cultivation with G) KNO_3, H) serine and I) the two-step cultivation before and after the addition of serine. Bioreactor conditions: pH 4.5, 30 °C, 800 rpm.

The cultivation with KNO_3 as sole nitrogen source reached carbon depletion approx. 75h, however CO_2 production continued until 120h before a sudden drop in CO_2 was observed. DW accumulation and pigment production reached its maximum after 130h, after CO_2 production had dropped. The cultivation yielded around 5 g/L of DW and 0.35 g/L of red pigment mixture. During the cultivation pigment changed colour from orange (PP-O) to red (atrorosins) as carbon was depleted as seen in Figure C4D). Just as observed in the shake flask cultivation, a mixture of red pigments was produced (Figure C4G).

Cultivation with serine as the sole nitrogen source reached carbon depletion at 180 h, yielding 0.9 g/L of atrorosin-S and 6.5 g/L of biomass (Figure C4B)). While cultivation duration was much longer compared to KNO_3, most of this can be attributed to a very long lag phase of almost 80 hours. Atrorosin-S production increased with fungal growth during the entire time course of the fermentation and no PP-O was observed as seen in Fig. 4E), and it is noteworthy to state that there was minimal atrorosin impurity as seen in Figure C4H).

Initial conditions of the two-step cultivation process included low amounts of potassium nitrate (2 g/L), which allowed the fungus to propagate. In this process, the start of the cultivation is similar to the control with a short lag phase of 30 hours before growth. The cultivation propagated for 55 hours producing the orange pigment PP-O shown in Figure C4I). As indicated from the CO_2 trace a limitation was reached at around 55 hours, and growth entered stationary phase. Based on the low amounts of potassium nitrate compared to the control, it was assumed that the nitrogen was depleted. At this time point, serine was added to the cultivation at a concentration of 1 g/L serving as new sole nitrogen source. Growth of the fungus continued and serine was incorporated into the PP-O azaphilone core structure, making atrorosin-S as seen in Figure C4F & C4I). At the end of the cultivation, all PP-O was converted into atrorosin-S as can be seen on the UV-chromatogram in Figure C4I). The two-step cultivation yielded 0.9 g/L of atrorosin-S, and 7.4 g/L biomass. Both, biomass accumulation and pigment production peaked at the time of carbon depletion at approx. 100h.

During the first phase of the cultivation on KNO_3 two isomers of PP-O were produced seen in Figure C4I). After the cultivation was switched to serine as nitrogen source, both isomers of PP-O were converted into only one isomer of atrorosin-S. This suggested a stereospecific conversion of PP-O to atrorosin-S by an enzyme. The peak at 8 minutes in the chromatogram corresponded to atrorosin-S confirmed by mass spectrometry ((*Isbrandt et al.* in progress) and the two peaks at 11 minutes corresponded to the *cis*- and *trans*- isomers of PP-O confirmed by mass spectrometry ((*Isbrandt et al.* in progress).

vitro synthesis was not affected by the L- or D-form of the amino acid, as both enantiomers from serine were successfully incorporated and both resulted in two isomers of atrorosin-S.

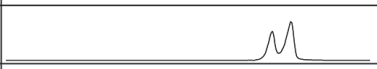
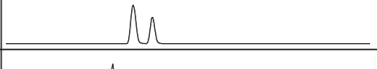
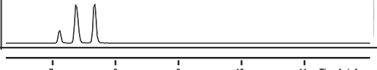
Amino Acid precursor	M (Da)	synthesized Atrorosin	UV- VIS- Chromatogram at pH 9	Color after reaction
	413.1595	control PP-O		
L-Serine	500.1915	Atrorosin-S		
D-Serine	500.1915	Atrorosin-S		
L-Glutamate	542.2021	Atrorosin-E		
L-Glutamine	541.2189	Atrorosin-Q		
L- Tryptophan	599.2388	Atrorosin-W		
L- Ornithin	527.2388	Atrorosin-Orn		
			7 8 9 10 11 Time [min]	

Figure C 5 UV-VIS- chromatograms (520 nm ± 10) and corresponding masses of the 6 synthesized analogues and the precursor PP-O at basic conditions. Elemental composition calculated from LC-HRMS (data not shown)

Glutamine and tryptophan, which did not lead to high atrorosin production in shake flask experiments, could however be used as precursors for atrorosin synthesis *in vitro*. Similarly, these results demonstrate that the non-proteinogenic amino acid ornithine could be incorporated *in vitro*. PP-O was under basic conditions instantly converted fully to the corresponding atrorosin, with the exception of glutamine where a small amount remained. Both isomers of PP-O were equally well converted and resulted in two isomers of the atrorosin. The two isomers of tryptophan co-eluted. Ornithine, which contains two primary amines, led to four isomers, of which two were also co-eluting.

Test for presence of Citrinin and other mycotoxins

Other azaphilone producers such as various *Monascus* species are known to also produce the mycotoxin citrinin, making them unsuitable for use in European and American food products. Citrinin was not detected in any of the pigment extracts from *T. atroroseus*. Figure C6 shows extracted ion chromatograms (EIC's) of citrinin (m/z = 251.0290) of the one- and two-step

production procedures with serine, as well as of the control process using potassium nitrate as the nitrogen source. It clearly demonstrates that citrinin is not produced under any of the three cultivation conditions.

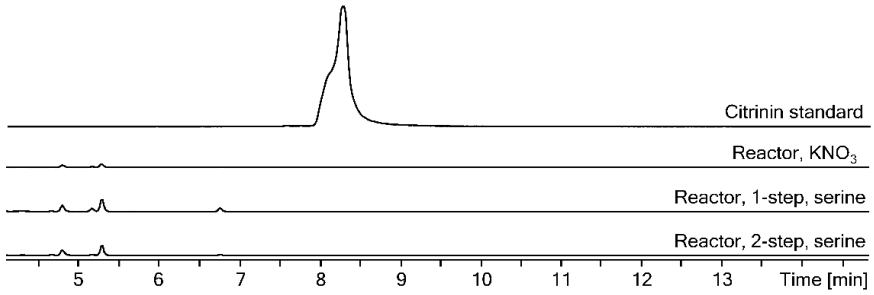


Figure C 6 Extracted ion chromatograms of citrinin (m/z = 251.0290) for an authentic standard versus the one- and two-step cultivation, as well as a for a standard procedure using potassium nitrate as the nitrogen source.

Discussion

The aim of this study was to investigate the effects of nitrogen sources on pigment biosynthesis in submerged cultivation of *T.atroroseus* and to design a process where specific pure pigments could be produced. Finally the mechanism of incorporation of amino acid into the core structure of atrorosins was investigated.

Influence of nitrogen source on pigment Biosynthesis

Submerged cultivation of *T. atroroseus* in shake flasks with different amino acids as sole nitrogen source, clearly shows that biomass accumulation and pigment production are not correlated (Figure C2). While all but two amino acids could be used for propagation of the fungus but only five amino acids promoted high amounts pigment. Individual use of proline, lysine, asparagine and tryptophan as the sole nitrogen source, did not result in pigment biosynthesis. We speculate that proline could not be incorporated into the pigment core structure because it is a secondary amine, as primary amines have been reported necessary for incorporation.

The differences in pigment yields across the amino acids could be affected by the dynamic environment in shake flasks which have no pH control, suboptimal mixing and limited oxygen saturation throughout cultivation. The final pH of the cultures varied greatly, likely due to the different nature of amino acids and the way they are metabolized and their charge (Data not shown). As reported in previous studies (Carels, 1979; Carels & Shepherd, 1978; Méndez,

Pérez, Montañéz, Martínez, & Aguilar, 2011), it was already hypothesized from the shake flask studies in this work, that pH is an important parameter for pigment biosynthesis but the optimal pH for pigment production does not necessarily correspond to the optimal pH for fungal growth (Santos-Ebinuma et al., 2014). To assure reproducible pigment production on different nitrogen sources, cultivation of *T.atroroseus* in controlled bioreactors with pH control was essential.

Analysis of the produced pigments, regardless of the amino acid as nitrogen source, demonstrates that cultivations with individual nitrogen sources produced their respective amino acid derivatives of PP-O. Interestingly, absorbance spectra of all amino acid derivatives were identical, with absorbance optimum of 510 nm. This is in contrast to Jung *et al.* 2003, who reported that amino acids like serine and glutamine would promote red colour while phenylalanine, isoleucine, leucine and valine would promote orange and yellow colour hues (Jung et al., 2003). We suspect that the orange colour of their supernatant was due to incomplete incorporation of the respective amino acids, and were in fact due to the presence of PP-O, monascorubramine, and other orange and yellow pigments in their samples. In their experiments, Jung *et al.* used a *Monascus* sp., which was cultivated with both, amino acids and low amounts of ammonium nitrate. Depletion of ammonium nitrate was not reported, suggesting that the orange colour was caused by ammonium and not by the amino acid derivative. Several studies demonstrated that the use of ammonium nitrate resulted in the formation of orange pigments by *Monascus* sp (Lin & Demain, 1995). By cultivation with amino acids as sole nitrogen source we have successfully avoided this phenomenon of cocktail pigments yielding pure atrorosins which all have a red chromophore.

Process protocol for high purity yielding azaphilone production

Building on the ability to produce pure atrorosins in shake flasks, two experimental procedures were hypothesized and tested in 1L bioreactors. First, a one-step cultivation using only serine as nitrogen source was tested, and secondly a two-step process, where an initial amount of potassium nitrate was used for biomass formation was followed by addition of serine to induce production of atrorosin-S. Both of these two novel methods successfully tailor the production to make pure atrorosin-S. Both, the one- and the two-step cultivation methods were conducted at a pH of 4.5 and resulted in significantly improved yields in regard to purity and quantity of atrorosin- S compared to other inorganic nitrogen sources. Comparison to control cultivations with potassium nitrate as nitrogen source, HPLC analysis demonstrate a mixture of different red atrorosins and PP-V (Table C1), whereas cultivations with serine

as sole nitrogen source or induced at potassium nitrate depletion, only atrorosin-S was detected in the fermentation broth. All previously reported cultivation protocols in the literature on both, *Monascus* or *Talaromyces*, resulted in the production of a pigment cocktail probably incorporating any molecules containing amino-groups. When providing only one amino-containing molecule in excess, this was preferred in the incorporation of PP-O.

Mechanism of amino acid incorporation

It is highly intriguing that only a subset of amino acids was incorporated into the core of PP-O when cultivated in shake flaks. Besides proline not having a primary amine, we expected amino acids such as asparagine, glutamine, tryptophan and lysine to be incorporated into the core structure. To address this, the precursor PP-O was purified for *in vitro* synthesis of atrorosins by addition of amino acids.

Glutamate, L-serine, and ornithine were used as positive controls, whereas glutamine, tryptophan, and D-serine were tested to evaluate incorporation. PP-O was reacted with the respective amino acid in a basic aqueous solution at pH 9. As demonstrated in Figure C5, addition of amino acids successfully incorporated into PP-O producing the respective atrorosins. It has been reported in other studies that *in vitro* conversion from orange into red *Monascus* pigments can be done (Jang, Choe, & Shin, 2014; Jung et al., 2003; Xiong, Zhang, Wu, & Wang, 2014), however *in vitro* synthesis of PP-V by addition of PP-O with glutamine in water has so far not been successfully conducted (Kojima et al., 2016). Xiong *et al.* report that a pH above 4 is critical for incorporation of amino acid both *in vitro* and *in vivo* (Xiong et al., 2014). Our results support that transamination is favoured at pH conditions above 4 and *in vitro* synthesis was successfully demonstrated at pH 9.

The results of this study are the first step in understanding the mechanism of amino acid incorporation into PP-O. PP-O as produced in the bioreactor exists as two isomers but subsequent addition of amino acid to the cultivation as seen in bioreactor converts PP-O to the respective atrorosin but only in one isomer form. Similar, in all one-step cultivation, both in bioreactors and shake flasks, only one atrorosin isomer was detected. These results suggest an enzymatic conversion of PP-O into the specific atrorosins under presence of the fungus. This hypothesis is in accordance with the literature (Kojima et al., 2016). However, *in vitro* incorporation of amino acids in PP-O into atrorosins, the two isomers of PP-O were converted into two entiomers of the respective atrorosins at pH 9, with the exception of tryptophan. The resulting two isomers of atrorosins *in vitro* and the only one isomer *in vivo*, further suggests that *in vivo* incorporation of amino acid into PP-O is enzymatic. We speculate that

amino acids which were not incorporated or minimally incorporated in the shake flask experiment as seen in Figure C2, is due to poor metabolisation of the respective amino acids leaving little free amino acid available for incorporation into atrorosins, or the respective amino acids alter the environment significantly, such as pH, as not to allow for precursor production.

Concluding remarks

Talaromyces atroroseus is a promising candidate for the production of novel natural pigments for industrial applications, as it excretes large amounts of water-soluble pigment into the medium. Current cultivation protocols with *T. atroroseus* use potassium nitrate, ammonium sulphate, or complex nitrogen sources and result in a mixture of different azaphilone pigments. We have in this study successfully demonstrated that by using a single amino acid as the sole nitrogen source, only one type of pigment is produced, namely the atrorosin amino acid derivative. This process allows for the desired amino acids derivative to be tailor-made by choice of amino acid. The high degree of purity of the atrorosin in the fermentation broth greatly eases downstream processing and purification, which are a necessity if this is to be industrially relevant for the food ingredient industry (Tolborg, Isbrandt, Larsen, & Workman, 2017).

Another important matter for the production of azaphilone pigments is safety, and citrinin has been widely associated with *Monascus* pigments. As seen in this study *T. atroroseus* does not produce citrinin under the cultivation processes described here, which greatly benefits *T. atroroseus* as an industrial fungal cell factory for pigment production. Production of pure products has priority for approaching regulatory path for approval of novel natural colorants for foods. In this study, two cultivation methods for production of a class of new compounds, atrorosins, in high purity was presented. For industrial relevance, the two-step cultivation method is to be preferred as it is cheaper in terms of nitrogen source, because only 2 g/L of amino acid compared was used compared to 10 g/L from the one-step cultivation. Furthermore, the two-step cultivation is shorter by 80 h. To further increase the industrial potential, a pre-culture could be implemented into the process potentially further reducing cultivation time (Arai et al., 2012; Jung et al., 2003; J Ogihara et al., 2000).

List of abbreviations:

PP-O	Penicillium purpurogenum- orange
PP-V	Penicillium purpurogenum- violet
PP-R	Penicillium purpurogenum- red
PP-Y	Penicillium purpurogenum- yellow

LCMS	Liquid chromatography mass spectrometry
DW	Dry weight

References

Amchova, P., Kotolova, H., & Ruda-Kucerova, J. (**2015**). Health safety issues of synthetic food colorants. *Regul. Toxicol. Pharmacol.*, *73*(3), 914–922.

Arai, T., Koganei, K., Umemura, S., Kojima, R., Kato, J., Kasumi, T., & Ogihara, J. (**2013**). Importance of the ammonia assimilation by Penicillium purpurogenum in amino derivative Monascus pigment, PP-V, production. *AMB Express*, *3*(1), 19.

Arai, T., Kojima, R., Motegi, Y., Kato, J., Kasumi, T., & Ogihara, J. (**2015**). PP-O and PP-V, Monascus pigment homologues, production, and phylogenetic analysis in Penicillium purpurogenum. *Fungal Biol.*, *119*(12), 1226–1236.

Arai, T., Umemura, S., Ota, T., Ogihara, J., Kato, J., & Kasumi, T. (**2012**). Effects of Inorganic Nitrogen Sources on the Production of PP-V [(10Z)-12-carboxyl-monascorubramine] and the Expression of the Nitrate Assimilation Gene Cluster by Penicillium sp. AZ. *Biosci. Biotechnol. Biochem.*, *76*(1), 120–124.

Arunachalam, C., & Narmadhapriya, D. (**2011**). Monascus fermented rice and its beneficial aspects: A new review. *Asian J. Pharm. Clin. Res.*, *4*(1), 29–31.

Broder, C. U., & Koehler, P. E. (**1980**). Pigments produced by Monascus purpureus with regard to quality and quantity. *J. Food Sci.*, *45*(3), 567–569.

Carels, M. (**1979**). The effect of changes in pH on phosphate and potassium uptake by Monascus rubiginosus ATCC 16367 in submerged shaken culture. *Can. J. Microbiol.*, *25*.

Carels, M., & Shepherd, D. (**1978**). The effect of pH and amino acids on conidiation and pigment production of Monascus major ATCC 16362 and Monascus rubiginosus ATCC 16367 in submerged shaken culture. *Can. J. Microbiol.*, *24*(11), 1346–1357.

Dapson, R. W. (**2007**). The history, chemistry and modes of action of carmine and related dyes. *Biotech. Histochem.*, *82*(4), 173–187.

Dufossé, L. (**2006**). Microbial production of food grade pigments. *Food Technol. Biotechnol.*, *44*(3), 313–321.

Dufossé, L., Fouillaud, M., Caro, Y., Mapari, S. A., & Sutthiwong, N. (**2014**). Filamentous fungi are large-scale producers of pigments and colorants for the food industry. *Curr. Opin. Biotechnol.*, *26C*, 56–61.

Frisvad, J. C., Yilmaz, N., Thrane, U., Rasmussen, K. B., Houbraken, J., & Samson, R. (**2013**). Talaromyces atroroseus, a New Species Efficiently Producing Industrially Relevant Red Pigments. *PLoS One*, *8*(12), e84102.

Gao, J., Yang, S., & Qin, J. (**2013**). Azaphilones: Chemistry and Biology. *Chem. Rev.*, *113*(7), 4755–4811.

Jang, H., Choe, D., & Shin, C. S. (**2014**). Novel derivatives of monascus pigment having a high CETP inhibitory activity. *Nat. Prod. Res.*, *28*(18), 1427–1431.

Jung, H., Kim, C., Kim, K., & Shin, C. S. (**2003**). Color characteristics of monascus pigments derived by fermentation with various amino acids. *J. Agric. Food Chem.*, *51*(5), 1302–1306.

Kojima, R., Arai, T., Matsufuji, H., Kasumi, T., Watanabe, T., & Ogihara, J. (**2016**). The relationship between the violet pigment PP-V production and intracellular ammonium level in Penicillium purpurogenum. *AMB Express*, *6*(1), 43.

Lin, T. F., & Demain, A. L. (**1991**). Effect of nutrition of Monascus sp. on formation of red pigments. *Appl Microbiol Biotechnol*, *36*, 70–75.

Lin, T. F., & Demain, A. L. (**1995**). Negative effect of ammonium nitrate as nitrogen source on the production of water-soluble red pigments by Monascus sp. *Appl Microbiol Biotechnol*, *43*, 701–705.

Lin, T. F., Yakushijin, K., Bt, G. H., & Demain, A. L. (**1992**). Formation of water-soluble Monascus red pigments by biological and semi-synthetic processes, *9*, 173–179.

Liu, B. H., Wu, T. S., Su, M. C., Ping Chung, C., & Yu, F. Y. (**2005**). Evaluation of citrinin occurrence and cytotoxicity in Monascus fermentation products. *J. Agric. Food Chem.*, *53*(1), 170–175.

Mapari, S. A. S., Meyer, A. S., Thrane, U., & Frisvad, J. C. (**2009**). Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale. *Microb. Cell Fact.*, *8*, 24.

Méndez, A., Pérez, C., Montañéz, J. C., Martínez, G., & Aguilar, C. N. (**2011**). Red pigment production by Penicillium purpurogenum GH2 is influenced by pH and

temperature. *Biomed & Biotechnol*, *12*(12), 961–968.

Ogihara, J., & Fujimotoz, Y. (**2000**). Biosynthesis of PP-V, a Monascorubramine Homologue by Penicillium sp. AZ. *J. Biosci. Bioeng.*, *90*(6), 2–4.

Ogihara, J., Kato, J., Oishi, K., & Fujimoto, Y. (**2001**). PP-R, 7-(2-hydroxyethyl)-monascorubramine, a red pigment produced in the mycelia of Penicillium sp. AZ. *J. Biosci. Bioeng.*, *91*(1), 44–47.

Ogihara, J., Kato, J., Oishi, K., Fujimoto, Y., & Eguchi, T. (**2000**). Production and structural analysis of PP-V, a homologue of monascorubramine, produced by a new isolate of Penicillium sp. *J. Biosci. Bioeng.*, *90*(5), 549–554. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16232908

Ogihara, J., & Oishi, K. (**2002**). OOEffect of ammonium nitrate on the production of PP-V and monascorubrin homologues by Penicillium sp. AZ. *J. Biosci. Bioeng.*, *93*(1), 54–59.

Papagianni, M. (**2004**). Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnol. Adv.*, *22*(3), 189–259.

Said, F. M., Brooks, J., & Chisti, Y. (**2014**). Optimal C:N ratio for the production of red pigments by Monascus ruber. *World J. Microbiol. Biotechnol.*, *30*(9), 2471–2479.

Santos-Ebinuma, V. C., Roberto, I. C., Francisca, M., Teixeira, S., Jr, A. P., Bioquímico-farmacêutica, D. D. T., Paulo, U. D. S., Biotecnologia, D. De, Lorena, E. D. E. De, & Paulo, U. D. S. (**2014**). Improvement of submerged culture conditions to produce colorants by Penicillium purpurogenum. *Brazilian J. Microbiol.*, *742*, 731–742.

Santos-Ebinuma, V. C., Roberto, I. C., Simas Teixeira, M. F., & Pessoa, A. (**2013**). Improving of red colorants production by a new Penicillium purpurogenum strain in submerged culture and the effect of different parameters in their stability. *Biotechnol. Prog.*, *29*(3), 778–785.

Scotter, M. J. (**2011**). Emerging and persistent issues with artificial food colours: natural colour additives as alternatives to synthetic colours in food and drink. *Qual. Assur. Saf. Crop. Foods*, *3*(1), 28–39.

Shi, K., Song, D., Chen, G., Pistolozzi, M., Wu, Z., & Quan, L. (**2015**). Controlling composition and color characteristics of Monascus pigments by pH and nitrogen sources in submerged fermentation. *J. Biosci. Bioeng.*, *120*(2), 145–154.

Stadler, M., Anke, H., Dekermendjian, K., Reiss, R., Sterner, O., & Witt, R. (**1995**). Novel bioactive azaphilones from fruit bodies and mycelial cultures of the ascomycete Bulgaria inquinans (Fr). *Nat. Prod. Lett.*, *7*(1), 7–14.

Tolborg, G., Isbrandt, T., Larsen, T. O., & Workman, M. (**2017**). Establishing novel cell factories producing natural pigments in Europe. In O. V. Singh (Ed.), *Biopigmentation and Biotechnological Implementation* (First Edit, pp. 23–60). Wiley, NJ, USA.

Wissgott, U., & Bortlik, K. (**1996**). Prospects for new natural food colorants. *Trends Food Sci. Technol.*, *7*(9), 298–302.

Woo, P. C. Y., Lam, C.-W., Tam, E. W. T., Lee, K.-C., Yung, K. K. Y., Leung, C. K. F., Sze, K.-H., Lau, S. K. P., & Yuen, K.-Y. (**2014**). The biosynthetic pathway for a thousand-year-old natural food colorant and citrinin in Penicillium marneffei. *Sci. Rep.*, *4*, 6728.

Xiong, X., Zhang, X., Wu, Z., & Wang, Z. (**2014**). Accumulation of yellow Monascus pigments by extractive fermentation in nonionic surfactant micelle aqueous solution. *Appl. Microbiol. Biotechnol.*, *99*(3), 1173–1180.

Yoshimura, M. (**1975**). Production of Monascus-Pigment in a Submerged Cultures. *Agr.Biol.Chem*, *39*(9), 1789–1795.

Declarations

Authors contributions

GT, AO, MW initiated and coordinated the entire project. All authors revised the manuscript. GT, AO, TI, TOL, MW conceived and designed the experiments. GT, AO and TI performed the experiments. GT, AO, TI, TOL analyzed the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data and materials are available from the corresponding authors on reasonable request.

Consent for publications

All co-authors have agreed to the content and form of the manuscript for publication.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix E
Supplementing material for Chapter 7

Table E1 Y_{XP} and final pH of the cultivation at 96 h of all tested amino acids as nitrogen sources and the control with KNO_3

Nitrogen source	Yield Y_{XP} (Intensity (AU/150µl)/ biomass (g/L))	pH of liquid medium at the end of the cultivation
Ala	0.03±0.01	4.9±0.73
Arg	0.02±0.02	4.0±0.55
Asn	0.10±0.03	5.2±0.31
Asp	0.60±0.52	5.3±0.14
Cys	0.03±0.62	4.8±0.10
Glu	0.88±0.07	5.7±0.01
Gln	0.00±0.04	3.8±0.08
Gly	0.04±0.15	5.4±0.11
His	1.91±0.06	4.2±0.08
Ile	0.50±0.02	4.1±0.02
Leu	0.72±0.05	3.9±0.05
Lys	0.16±0.03	4.9±0.02
Met	0.32±0.19	3.8±0.03
Phe	0.11±0.12	3.9±0.06
Pro	n.a.	4.5±0.07
Ser	3.13±0.02	4.5±0.13
Thr	1.63±0.03	4.8±0.06
Trp	n.a.	4.2±0.05
Tyr	0.41±0.03	3.3±0.03
Val	0.16±0.16	4.2±0.07
Ornithin	0.04±0.07	3.0±0.06
KNO_3 (control)	0.25±0.08	5.3± 0.03